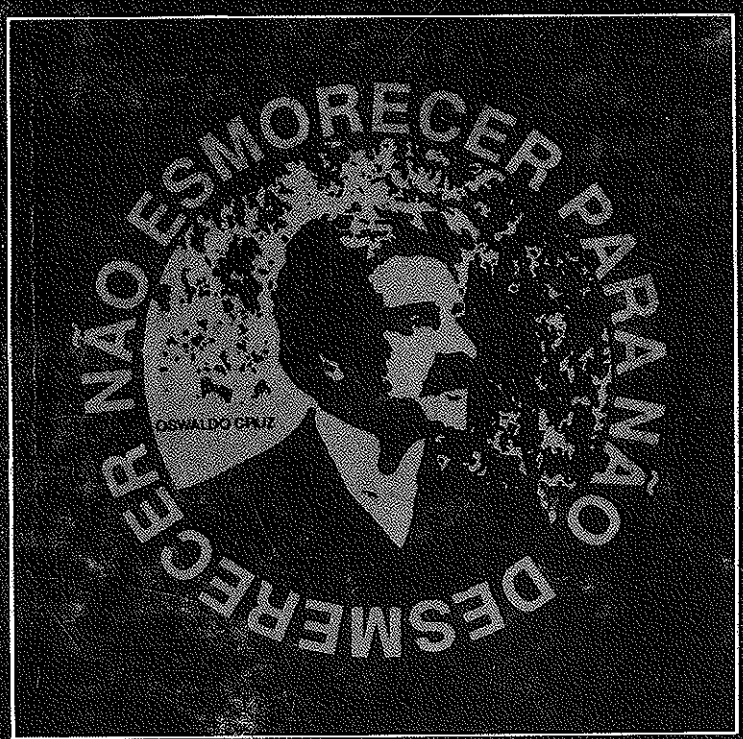
ISSN 0001-3714

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology



SBM Sociedade Brasileira de Microbiologia

São Paulo — Brasil

Volume 26 Número 3 Jul. - Set. 1995

FICHA CATALOGRÁFICA

Preparada pela Biblioteca do
Instituto de Ciências Biomédicas da Universidade de São Paulo

Revista de Microbiologia/Sociedade Brasileira de Microbiologia.
Journal of the Brazilian Society for Microbiology
— Vol. 26, nº 3 (jul/set 1995)
— São Paulo: SBM, [1970] -
v.:il; 27 cm

Trimestral
1970 - 1995, **3-26**
ISBN 0001-3714

1 . Microbiologia I. Sociedade Brasileira de Microbiologia

NLM-QW4

SCT/PR CNPq FINEP

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Publicação da Sociedade Brasileira de Microbiologia - São Paulo - Brasil
Publication of the Brazilian Society for Microbiology - São Paulo - Brazil

Filiado a / *Filiated to*:
IUMS - International Union of Microbiological Societies

Os artigos publicados na Revista de Microbiologia (Journal of the Brazilian Society for Microbiology) são indexados em:
Papers published in Revista de Microbiologia (Journal of the Brazilian Society for Microbiology) are indexed in:
Current Contents (USA); CNRS - Centre de la Recherche Scientifique (France); Chemical Abstracts Service (USA); Cambridge Scientific Abstract (USA); Commonwealth Mycological Institute (England); Hamdard National Foundation (Pakistan); IMLA - Index Medicus Latino Americano (Brasil); Institut Nauchtoi Informatsii (ex-URSS); Periodica (Mexico); Sumários Correntes Brasileiros (Brasil); UMI - University Microfilms International (USA).

Apoio Financeiro / *Financial support*: FINEP , FAPESP and CNPq.

Produzido por / *Printed by* WINNER GRAPH (phone: (011) 584.6023)

Sociedade Brasileira de Microbiologia
Av. Prof. Lineu Prestes 1374 - phone/fax 55-11-813-9647
05508-900 - São Paulo - SP
Brazil

REVISTA DE MICROBIOLOGIA
Depto. de Microbiologia – ICB II – USP
Av. Prof. Lineu Prestes, 1374 – Cid. Universitária
CEP 05508-900 – São Paulo - SP - BRASIL
Site: www.revmicro.cjb.net

COMUNICADO

Lamentamos informar a toda comunidade científica brasileira e internacional o inesperado falecimento da Diretora Executiva da Revista de Microbiologia, Dra. Maria Therezinha Martins. Seu amor e dedicação à Revista de Microbiologia e à Sociedade Brasileira de Microbiologia permanecerão na lembrança de todos aqueles que tiveram a feliz oportunidade de trabalhar a seu lado.

Informamos também que a partir de 1996 o novo Diretor Executivo será o Dr. Luiz R. Trabulsi, do Departamento de Microbiologia da Universidade de São Paulo.

Em vista do falecimento da Diretora da Revista de Microbiologia, algumas alterações administrativas tiveram que ser introduzidas, a saber:

- o novo Editor Seccional para a área de Microbiologia Ambiental é o Prof.Dr. Allen Norton Hagler, do Instituto de Microbiologia da Universidade Federal do Rio de Janeiro. Os demais Editores Seccionais permanecem os mesmos.

- ao invés de encaminhar os originais do trabalho para o Editor Seccional, os autores deverão enviar 3 cópias do manuscrito para o seguinte endereço:

Revista de Microbiologia
Av. Prof. Lineu Prestes, 1374
05508-900 - São Paulo - SP
Brasil

A Revista de Microbiologia encaminhará oa manuscritos para o Editor Seccional

COMMUNICATION

We are sorry to inform the Brazilian and the International scientific community about the passing away of Prof. Maria Therezinha Martins, Executive Director of Revista de Microbiologia. Her enthusiasm about her work and her dedication to Revista de Microbiologia and to the Brazilian Society for Microbiology will stay forever with those who had the opportunity to work with her.

We would also like to announce that from 1996 on the new Executive Director of Revista de Microbiologia will be Prof. Luiz Trabulsi, from the Microbiology Department at Universidade de São Paulo.

Some administrative modifications have been introduced:

1 - The new Section Editor for Environmental Microbiology is Prof. Allen Norton Hagler, from Instituto de Microbiologia at Universidade Federal do Rio de Janeiro. The other Section Editors remain the same.

2 - Instead of submitting the manuscript to the Section Editor, the authors are requested to send three copies of it to the following address:

Revista de Microbiologia
Av. Prof. Lineu Prestes 1374
05508-900 - São Paulo - SP
Brazil

Revista de Microbiologia will forward the manuscript to the correspondent Section Editor.

REVISTA DE MICROBIOLOGIA
Journal of the Brazilian Society for Microbiology
PUBLICAÇÃO DA SOCIEDADE BRASILEIRA DE MICROBIOLOGIA
Publication of the Brazilian Society for Microbiology
VOLUME 26 JULY-SEPTEMBER 1995 NUMBER 3
REV. MICROBIOL. (S.PAULO), 26(3)

CONTENTS-CONTEÚDO PÁG.

Rev. Microbiol., São Paulo, **26**(3): 151-159, 1995

MICROBIAL CELL IMMOBILIZATION APPLIED TO ALCOHOL PRODUCTION - A REVIEW

Angelo Maurício Lother[1]
Marilia Oetterer[2]

ABSTRACT

A review on microbial cell immobilization applied to alcohol production by continuous fermentation was made. The types of immobilization and the various supports were discussed. In the future, this kind of methodology will be important due to its economic advantages, particularly when starch is used as substrate.

Key words: microbial cell immobilization, continuous fermentation, alcohol production.

INTRODUCTION

The best example of production of an alternative liquid fuel as a reaction against the rise in oil prices can be found in Brazil. This solution derived from an old practice established back in the early thirties, when the Brazilian government decided to officialize the blending of ethanol into gasoline.

Any product composed of sugar or other carbohydrate is a potential raw material for ethanol generation. However, from an economical standpoint, it is necessary to consider the volume of its production, the agricultural and industrial yield and the cost of manufacture.

With the petroleum shortage, there has been revived interest in ethanol production via fermentation. The advantage of microbiological methods over chemical processes is that the raw material used is renewable. According to the U.S. Gasohol program at the end of 1981, 500 million gallons of ethanol were generated. The production increased to 1.8 billion gallons in 1985 and reached 9.0 billion gallons by 1995. In order to boost this program, the Administration provided loans and tax credits for ethanol made from renewable sources (17).

The announcement by the Brazilian government of a National Alcohol Production Program involving $400 millions of capital investment is the biggest single incentive to the production of alcohol by fermentation (1).

Among the various immobilized biocatalysts available, immobilized whole cell systems have received special attention. The advantages of whole (living) cells systems include their catalyzing ability to synthesize various useful and complicated chemicals by multi enzyme steps, and their regeneration activity that prolongs the catalytic life (8).

Ethanol is now produced in the United States by fermentation of grain, especially corn. Sweet potato has been considered as a good substrate for ethanol production. Another excellent substrate, which has received very little attention, is the Jerusalem artichoke tuber. These crops contains a high level of inulin that can be hydrolyzed mainly to D-fructose (14).

When raw materials containing polysaccharide are used for alcohol fermentation by yeast, they are first converted to fermentable sugars using enzymes (alpha amylase, commonly derived from *Bacillus*

[1] Eng. Agron. Mestrando. CNPq. Universidade de São Paulo, Escola Superior de Agricultura "Luiz de Queiroz", Departamento de Ciência e Tecnologia Agroindustrial.

[2] Profª.Drª. Universidade de São Paulo, Escola Superior de Agricultura "Luiz de Queiroz", Departamento de Ciência e Tecnologia Agroindustrial. Av. Pádua Dias, 11. Caixa Postal 09. CEP 13.418-900. Piracicaba - SP. Brasil.

strains and glucoamylase from *Aspergillus* strains), inorganic acids, irradiation, ultrasonic techniques and ball milling. Combinations of acid hydrolysis and physical extrusion have been used. However, acid hydrolysis requires corrosion-proof equipment, and yields undesirable byproducts. On the other hand, enzymes used for hydrolysis are slow and expensive. Physical treatments have also been used for pretreatment of cellulosic materials prior to enzyme action. High pressure in combination with heat and acid can improve the depolymerization of starch to fermentable sugars (11, 15).

A combined saccharification and fermentation process would eliminate the saccharification stage and could reduce fermentation costs. In addition, keeping the glucose at a low concentration would avoid enzymatic reversion and transglucosyl reactions that repolymerize glucose and decrease glucose yield. The *Aspergillus awamori* glucoamylase gene has been cloned into a laboratory yeast strain and a distiller's yeast strain to allow combined saccharification and fermentation of soluble starch. Direct fermentation of soluble corn starch to ethanol by addition of glucoamylase to the broth is a common practice in the industry. Furthermore, it has been demonstrated in a mixed culture of *Zymomonas mobilis* and *A. awamori*.

Among the efficiency processes, the continuous fermentation technique has been extensively investigated, but it presents some problems on process stability and production efficiency. In this respect, the recently developed immobilized microbial cell system looks attractive and promising for economical ethanol production, since continuous production by this system is superior to that by conventional fermentation processes (2, 12).

Enzymes can catalyze various reactions under mild conditions, and can efficiently produce useful compounds of specific complicated structures not easily synthesized by chemical processes. However, enzymes are generally not so stable as to maintain their catalytic activity for a long period. Furthermore, it is very difficult to recover enzymes from a reaction mixture for reuse on continuous reactions. To immobilize intracellular enzymes it is first necessary to extract them from the cells. In order to avoid this procedure, the technique of direct immobilization of whole microbial cells was studied. In a batch ethanol process, fermentation is responsible for 80% or more of total capital costs. Consequently, a number of continuous processes have been developed and applied both in alcohol beverage manufacture and in industrial ethanol production, where costs are largely determined by the price of fermentable carbohydrates. Therefore, much attention is currently focused on utilization of inexpensive waste materials and byproducts such as cellulose and whey as feed stock and on the improvement of overall economics of the process. The microorganisms that have been used are *Saccharomyces sp, Saccharomyces amuraceae, Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Kluyveromyces fragilis* and *Z. mobilis* (3).

The methods of immobilization may use inert carrier, porous glass, silicate, PVC, porous brick, kieselguhr, glutaraldehyde, entrapment in polyethylene, cellophane, Ca^{2+} alginate gel beads, K-carrageenan, etc (9, 18,19).

Definition and Advantages of Immobilized cells

An immobilized cell is defined as a cell, or a remnant thereof, that by natural or artificial means is prevented from moving independently from its neighbors to all parts of the aqueous phase of the system under study (35).

Immobilization is a generic term used to describe the retention of a biologically active catalyst within a reactor or analytical system. The biocatalyst, be it a single enzyme, mixture of enzymes or enzymes contained inside a living cell, is confined within or on a support material. The immobilized complex takes on the physical characteristics of the support while retaining the basic biochemical activity of the free catalyst, thus improving the handling properties of the catalyst and its bioconversion efficiency (31).

Immobilization is the application of heterogeneous catalysis to biological systems. Most existing biochemical processes and techniques use a soluble enzyme or finely divided cell monoculture to convert a substrate (low value material or measurable parameter). Immobilization involves confining the soluble protein catalysts or individual, neutral buoyancy cells, in a reactor system which can repeatedly treat fresh charges of solutions. The immobilized material might be retained in a column reactor through which a fluid is passed continuously. In this case, the solutions

are converted as they pass through the bed and emerge continuously as a catalyst-free product. Alternatively, immobilization may aim at providing a catalytic material which can be moved repeatedly from one container of process fluid to another, converting substrate to product on a batch principle (31).

Many useful compounds especially produced through a fermentation method are normally formed by multi step reactions, catalyzed with many kinds of enzymes from living microbial cells. Additionally, these reactions often require generation of ATP and a coenzyme. If immobilized cells are kept in a living state, they may be used for these multi enzyme reactions. Immobilized microbial cells utilized as a multi enzyme system may be classified into three physiological states: dead, living and growing state. So, the state most suitable for application purposes must be selected (2).

Immobilization provides an insoluble complex on a specialized module through which fluids can pass easily, transforming substrate to product in a controlled enzymatic reaction and facilitating the removal of the catalyst from the product as it leaves the reactor. Its main aim is to apply the benefits of heterogeneous cell suspensions used routinely in academic bioscience and industrial biotechnology (31).

Microbial enzymes can be extracellular, excreted from cells into the broth, and intracellular, retained within the cells during cultivation. In order to utilize intracellular enzymes, it is necessary to extract them from the microbial cells. These extracts are generally unstable and often undesirable for practical use as immobilized enzymes. Many useful chemical substances have to be produced by fermentation when the catalytic activities of multi enzyme systems of microorganisms are to be employed. Direct immobilization of whole microbial cells can solve these problems (2).

The immobilized microbial cells can be growing, resting or dead, yet the enzymes have to be kept in the active state (3).

Processes based on immobilization of living microbial cells have several advantages compared with traditional fermentation by free cells. The reaction rate is accelerated due to increased cell density, it is possible to achieve higher specific product yield, to conduct operation on a continuous basis at a high dilution rate without a washout eliminate costly fermenter design, and to provide better and easier control of fermentation processes. Such systems are better even when compared with more recent immobilized enzyme processes, since neither extraction nor purification of the enzyme from the microbial cell is necessary. Furthermore, the loss of enzyme activity is reduced, and the preparation and utilization of microbial cells are an easy task compared with the handling of immobilized enzymes. Enzyme stability is higher in whole cells. When cofactors are required, the use of cells is preferred to enzymes, since cells may regenerate the cofactors. Cells can be attached without significant loss of catalytic activity and their operational and storage stability is high. Finally, the risk of contamination is reduced due to fast dilution rates and high yeast cell densities (13, 17, 37).

However, some limitations still remain: a) the cells may contain other enzymes that catalyze side reactions or enzymes that change the studied product, b) protease action may denature some enzymes. However, these unfavorable reactions can be often avoided by heat-treatment, acid-treatment or chemical-treatment before or after immobilization of microbial cells (7).

With respect to the applications of immobilized microbial cells, workers in the field first succeeded in the industrialization of this type of system with the continuous production of L-aspartic acid in 1973. Afterwards, the system was applied to the industrial production of L-malic and L-alanine. The production systems for 6-APA and high fructose syrups on an industrial scale can be added to these examples (3).

So far, immobilized microbial cells have been primarily used in a single step enzyme reaction. For the unit production of a desired compound, the required volume of fermentation broth is much smaller in the case of continuous reactions using immobilized cells as compared to conventional batch fermentation. Thus, the continuous process may reduce plant pollution problems (5).

Potential advantages of immobilized whole cell systems over controlled fermentations are: a) placement of fermentation on a heterogeneous catalysis design basis; b) higher product yields; c) ability to conduct continuous operations as opposed to traditional batch fermentation; d) operation at high dilution rates without washout; e) ability to recharge the system by inducing growth and reproduction of resting cells; f) decrease or

elimination of lag and growth phases for product accumulation associated with the non-growth phase of fermentation; and g) possibility of accelerated reaction rates due to increased cell density (35).

The overall rationale for whole cell immobilization is outlined as follows: a) obviation of enzyme extraction/purification; b) generally higher operational stability, c) lower effective enzyme cost; d) high yield of enzyme activity on immobilization; e) cofactor regeneration; f) retention of structural and conformational integrity; g) greater potential for multi step processes; h) greater resistance to environmental perturbations (35)

Categories of Immobilized Cells

The techniques of immobilization can be classified into three categories: carrier-binding, cross-linking and entrapping. The carrier-binding method is based on direct binding of cells to water-insoluble carriers by physical adsorption, ionic bonds or covalent bonds. As carriers, water insoluble polysaccharide (cellulose, dextran and agarose derivatives) proteins (gelatin and albumin) synthetic polymers (ion-exchange resins and polyvinyl chloride) and inorganic materials (brick, sand and porous glass) are used. Cells can be immobilized by cross-linking with bi or multifunctional reagents such as glutaraldehyde, toluene di isocyanide and others. The method of directly entrapping cells into polymer matrices has been intensively investigated. The matrices are: collagen, gelatin, agar, alginate, carrageenan, cellulose, tri acetate, polyacrylamide, epoxy resin, photo-cross-linkage resin, polyester, polystyrene and poly urethane (5, 16).

The most common approach in whole cell immobilization is using the course of carrier preparation. The methods are entrapment and encapsulation. Due to the size of whole cells, it is rather simple to prepare networks of such porosity that complete cell retention is guaranteed, and transport processes for substrates and products are fast enough to obtain a high efficiency of catalytic activity. The main problem to be solved is to match the conditions of carrier preparation to the physiological requirements for the retention of both enzymatic activity and cell viability. Immobilization by entrapment, due to its simplicity and flexibility, has now become by far the most extensively used immobilization technique (16).

The most widely used method is entrapment and the most often chosen substances are polyacrylamide, calcium alginate and carrageenan. We have to distinguish between inorganic and organic materials, and between synthetic materials of different levels of sophistication. In the group of inorganic substances, we have bricks, sand particles, ceramics, metal hydroxides and controlled-pore glass. Organic materials used are wood, chips, anthracts, collagen, cellulose, carrageenan, alginate and albumin. Synthetic organic materials are polyvinyl chloride, poly propylene, polyacrylamide, ion-exchange resins, epoxies and poly urethane. The method of entrapment is a domain of organic polymers, mainly alginate and carrageenan (16, 29).

Studies on the utilization of ion exchange resins as agents for whole-cell immobilization and the use of continuous flow packed-bed bioreactor were made. The efficacy of these systems can be seen when applied to ethanol production by yeast cells and by the bacterium *Z. mobilis*. A recent study proposed that nutrient levels (particularly phosphate) may be responsible for filament formation by *Z. mobilis*. This microorganism immobilized on microporous ion exchange resins has previously been shown to allow the attainment of high ethanol productivity in a packed-bed bioreactor. The formation of bacterial filaments after several days of continuous operation, however, resulted in excessive pressure increases across the reactor bed (6).

Ethanol production by entrapped and free *S. cerevisiae* has been compared in particular conditions. The total amount of produced ethanol was lower for free cells (472 and 388 mM/L) and the production rate was 5 times higher with entrapped cells. When cells are covalently linked to porous glass, the activity is decreased, but the yield is always higher than with free cells (7).

Gel entrapment, because of its mildness, ease of operation and wide applicability, has been one of the most studied immobilization techniques. It is the trapping of cells within a three-dimensional matrix, where the matrix pore size is smaller than the cell size. Materials of this kind range from natural polysaccharides and proteins to purely synthetic polymers such as polyacrylamide (34).

Adsorption of cells onto a solid surface is probably the mildest of the cell immobilization

techniques. It is one of the cheapest methods and thus particularly useful for industrial processing. In adsorption, there is generally an initial weak attachment of the cells, which can be easily reversed. This is followed by the development of stronger (multiple attachment) binding. Often the extracellular material produced by the cells is important in fixing them to the adsorption substrate, and this may be followed by natural entrapment of the cells in the biopolymer matrix. The physical form of the support has received considerable attention, particularly with regard to porosity and the shape of the material (34).

A summary of the categories, supports and respective reactions utilized on immobilization techniques is given below:

Methodology; ethanol production with immobilized cells

A variety of systems have been proposed for the rapid and continuous production of ethanol by fermentation. Many of these systems are quite promising and have addressed some of the most important techno-economic considerations on the bioconversion of carbohydrates to ethanol, namely: high ethanol productivity, high final ethanol concentration, near complete utilization of substrate and ease of operation (22, 26).

The most recent advance in alcohol production technology is the application of immobilized cells to continuous column reactors. This approach combines several advantages of the systems already discussed. For example, a high cell concentration can be obtained, the reaction rate is accelerated and operation can be performed at a high dilution rate without a washout. The end product inhibition is eliminated since alcohol is constantly removed. Anaerobic conditions can be attained because cells are entrapped into a gel matrix, and, in contrast with already described processes, costly equipment design such as fermenter, agitators, etc are eliminated (17).

The first industrial application of immobilized enzymes in the world goes back to 1969, when immobilized amino acylase was used for the continuous production of L-amino acids from acetyl-DL-amino acids. K-carrageenan is one of the most suitable polymers for immobilization of microbial cells when compared with many synthetic and natural polymers utilized as matrix for entrapping enzymes and microbial cells into gel lattices (2).

Difficulty is sometimes experienced in distinguishing between conventional fermenters and immobilized cell reactors. A fermenter, regardless of its exact type, is a culture vessel designed firstly to increase the biomass of the active organisms with which it has been inoculated. This growth may accompany or precede the conversion of growth substrate into some useful metabolic product. In the batch mode of operation fermentation is terminated at a time considered appropriate, and the cycle of inoculation, growth and harvesting repeated. In the continuous mode of operation, growth related substances or biomass itself can be readily produced but many metabolites of economic interest are often not produced in sufficient quantities. Much of the industrial and academic research into enzyme and cell immobilization has focused on the search of extended life biocatalysts. It has been found by experiment that free cells grown in a fermentermay have their useful biocatalytic life considerably extended by immobilization. Cells returned in the immobilized state to a vessel essentially similar to the original

CATEGORIES	CARRIERS	REACTIONS
Carrier binding	cellulose, dextran, agarose, gelatin, albumin, polyvinyl chloride, brick, sand, porous glass.	physical absorption ionic, covalent bonds
Cross-linking	glutaraldehyde, toluene, di isocyamide. collagen, gelatin, calcium alginate, carrageenan, agar, cellulose, triacetate, polyacrylamide, epoxy resin, polyester	bi, multi reagents entrapping

fermenter make up an immobilized cell reactor (34).

In France, alcohol production was studied using *S. cerevisiae* immobilized by adsorption on brick or polyvinyl chloride chips. The maximum alcohol production was 120 grams per liter, and 46% of the conversion of sugar to alcohol was achieved. In Japan, alcohol production using *S. carlsbergensis* cells immobilized by entrapment into K-carrageenan gel was studied. In this case, continuous alcohol production at the level of 100 mg/ml was observed and the conversion of glucose to ethanol was 100% of the theoretical yield (17, 32).

The ethanol production by *S. cerevisiae* immobilized in hollow-fiber membrane bioreactor has been studied. *S. cerevisiae* ATCC 4126 was grown within the macroporous matrix of asymmetric-walled polypropylene hollow-fiber membranes. In a patent assigned to the United States Department of Energy, a column reactor was filled with conventional distillation-column packing such as berl saddles coated with gelatin in the presence of polyelectrolyte and a cross-linking agent. Stable yeast populations could be maintained for extended periods by restricting nutrients and by addition of a small quantity of membrane-disrupting detergent to the substrate feed, to make dead cells available as nutrient for the remaining yeast population (10, 19).

Alcohol production from starch bioconversion has been studied using *Aspergillus niger* and *Bacillus subtilis* as the first step, coupled to *S. cerevisiae* which converts glucose produced from the first microorganisms into ethanol. This research was made on a column bioreactor as a continuous system and using alumina beads as solid support. The flux of the feed stock was 45 $ml.h^{-4}$. After 48 hr the starch bioconversion to glucose was 50% and the efficiency of the process was 94.90% (20, 21, 27 and 28).

More recently, researches are using mineral charcoal as carrier as well as irradiated starch on enriched feed stock and malt extract added to the yeast medium, in order to improve the performance of the microorganism in the system and to take economic advantage.

Immobilization with Carrageenan.

Carrageenan is a readily available nontoxic polysaccharide isolated from seaweed. It is composed of a unit structure of ß-D- galactose sulfate and 3,6- anhydrous-a-D-galactose (5)

Ethanol is produced from glucose via a series of multi step enzyme reactions involving ATP and NADH regeneration systems. Ethanol can be continuously and efficiently produced by using living yeast cells immobilized with carrageenan (30). The immobilization of the yeast, *S. carlbergensis* can be done with a carrageenan solution with immobilization in the form of beads. A layer of yeast cells will be generated depending on the diffusion of nutrients into the gel. That is, the cell layer is formed as a result of selection of suitable environmental conditions by microbial cells. The steady state of number of living cells and ethanol production in such system was maintained for longer than 30 days. Produced ethanol concentration was 50 mg/ml and residual glucose concentration was about 2 mg/ml. These values indicate that the conversion of glucose to ethanol was almost 100% of the theoretical yield, even at fast flow rates such as retention time of 1 hour. This technique is being improved, and it is now possible to produce continuously over 100 mg/ml of ethanol (2, 4).

Changes in the growing state of *Saccharomyces sp* IFO 2347 immobilized on carrageenan gel beads were investigated. By incubating the gel in nutrient medium, the weights of gel beads were found to increase and yeast cells increased by growth. Scanning electron microscopy of the yeast cells immobilized on gel revealed that colonies enlarged upon incubation were assembled near the gel surface (36).

Immobilized growing *Z. mobilis* cells fermented rapidly and efficiently on media containing 100 g/L fructose in a continuous reactor. A volumetric ethanol productivity of 94.8 $g/L.h^{-1}$ was achieved at a substrate conversion of 75.5%. Ethanol production was constant over a period of 55 days. Cells were first entrapped in K-carrageenan and then activated by incubation in the culture medium one or several times, in order to increase the cell concentration inside the gel. Typical values were in the range 29-54 g cell mass/L gel. This preparation was used in a continuous reactor (12).

Immobilization with Alginate

Calcium alginate, which is extracted from seaweed, is a linear copolymer of D- mannuronic and L-guluronic acid. It can be gelled by multivalent ions such as calcium and aluminum (5)

S. cerevisiae cells have been immobilized on

calcium alginate beads for use in the continuous production of ethanol. Yeast slurry containing 1.5% alginate (w/v) was added as drops to a 0.05 M $CaCl_2$ solution. Ethanol production rates as well as residual sugar concentrations were monitored at different feed stock flow rates(37).

S. cerevisiae cells, *Kluyveromyces marxianus* cells, inulase, glucose oxidase, chloroplast and mitochondria were immobilized on calcium alginate gels. Ethanol production from glucose solutions by an immobilized preparation of *S. cerevisiae* was demonstrated over a total of 23 days, and the half-life of such a preparation was shown to be about ten days. Immobilized *K. marxianus*, inulase and glucose oxidase preparations were used to demonstrate the porosity and retaining properties of calcium alginate gels. Calcium alginate immobilized chloroplast were shown to perform the Hill reaction. The maximum efficiency reached by the system was 90% of the theoretical maximum yield (13).

Ethanol production was studied in a column packed with immobilized living yeast cells. During continuous production of ethanol, the steady state was maintained for longer than 90 days. A stepwise increase in the concentration of glucose was found to be necessary in order to obtain a constant production of high concentrations of ethanol. A large amount of *S. cerevisiae* entrapped in calcium alginate gel was used to convert glucose to ethanol. *S. cerevisiae* cells immobilized on calcium alginate gel have also been used for continuous production of ethanol from cane molasses. In addition, *Z. mobilis,* which *has* several times higher ethanol productivity than yeast, was immobilized on calcium alginate gel and K-carrageenan gel. Both types of immobilized cells showed high ethanol productivity, yet there was a decline of 30% in activity after 800 hrs of operation. If a novel strain is constructed with resistance to higher concentrations of ethanol and/or glucose and an ability to grow well in the immobilization matrix, this immobilized biocatalyst system will become a more promising method for ethanol production (4).

The continuous production of ethanol from yeasts immobilized on pectin gel using a horizontal reactor designed to reduce the negative influence of released gas was studied. During the first 3 days, ethanol concentration in the effluent increased rapidly until a maximum of 70 g/L. The system then reached steady state, when productivity was 40 g ethanol/h.L^{-1}, estimated on the basis of a liquid working volume of 290 mL. During this period, the yield of ethanol was 0.49 g /g consumed sugars and the fermentable substrate utilization was about 90%. After approximately 15 days without significant variations, the performance of the system began to decrease. Thirty days after the start, the productivity of the assay was 40% lower. This gradual decrease in the fermenter's performance was in all probability due to the inhibitory effect of ethanol on fermentation and its negative action on yeast viability (25).

The yeast *Pachysolen tannophilus* was entrapped in calcium alginate beads to ferment D-xylose on a continuous basis at high cell densities. Under favorable operating conditions, cultures retained at least 50% of their initial productivity after 26 days of operation.The specific ethanol production rate was dependent on the substrate level in the fermenter, passing through an optimum when D-xylose concentration was between 28 and 35 g/L.*P. tannophilus* can utilize the pentose fraction unfermentable by ordinary brewers yeasts, adding at least 4.5 billion gallons of ethanol to the estimated annual production of alcohol from crop residues (33).

Immobilization with Glass, Silica and Alumina Beads.

Porous glass beads are used to immobilize *S. carlsbergensis* cells. If silica pores are large enough, adsorption occurs. On the other hand, activation of the silica by glutaraldehyde allows the cells to bind onto the support. In all cases, 15 minutes are sufficient for support saturation by microorganisms. The study of glucose fermentation by immobilized cells shows that immobilization modifies cell metabolism. Adsorption leads to an acceleration of metabolism, while a slowing down in cell activity follows covalent binding. Nevertheless, in both cases, the yield of ethanol from glucose convertion increases while the yield of carbon dioxide decrease. *S. carlsbergensis* was grown in an Erlenmeyer flask with Sabouraud liquid medium at 30°C under shaking. Cells were harvested by centrifugation (15 min at 4500 G) and washed three times by suspension in 300 mL 0,9% saline solution. The supports were porous silica beads, with diameter and specific area ranging from 100 to 200m and 6

to 445 mg/g, respectively. Cells and supports were suspended in 0.02 M citrate phosphate buffer, pH 5.0, and mixed for 1 hr at room temperature. Silica was decanted and rinsed five times with the same buffer (24).

Vertical and near horizontal (15° angle) packed-bed columns were compared for continuous ethanol fermentation using an alcohol and glucose tolerant *S. cerevisiae* strain immobilized onto channeled alumina beads (5.0×10^8 cells/g beads). Malt-glucose-yeast extract broth at 35°C containing 16.7% glucose was fed at a dilution rate of 3,1/h to the two horizontal columns (in series); the maximum ethanol productivity was 40.0 g/L.h^{-1}. Feed stock flow rate and other factors (temperature, pH, nutrients, and glucose levels) affected productivity. The immobilized-cell system showed operational stability for 3 months without plugging, and could be stored at least for one year with no loss of bioreactor performance. Scanning electron micrographs of the beads revealed large numbers of yeast-cells attached onto internal and external surfaces of the beads (9).

CONCLUSION

The production of alcohols using immobilized living microbial cells has been the subject of increased interest, and appears to be a promising technique for the industrial production of ethanol. The advantages of this methodology when compared with the traditional process are many, including high yields and reduced costs. With starch substrates, a combined saccharification and fermentation process could eliminate the saccharification stage and reduce fermentation costs when used in immobilization processes.

RESUMO

Utilização da técnica de imobilização de células microbianas para produção de álcool

Este trabalho se constitui em uma revisão sobre o emprego do método de imobilização de células microbianas no processo de fermentação contínua para a produção de etanol. Os vários tipos de imobilização e os suportes mais empregados foram discutidos. Futuramente este tipo de metodologia ocupará importante papel na fermentação industrial devido às suas vantagens econômicas, principalmente na obtenção de álcool a partir de substrato amiláceo.

Palavras chaves: imobilização de células microbianas, fermentação contínua, produção de álcool.

Summary of cell immobilization fermentation processes:

Microorganisms	Supports	General Characteristics
S.cerevisiae	poly propylene gelatin+polyelectrolyte	column reactor
S.carlbergensis	carrageenan continuous production.	30 days, 50mg/ml ethanol,
Z. mobilis	k-carrageenan productivity, 75.5% conversion	55 days, 94.8g/L.h^{-1}ethanol
S. cerevisiae	calcium alginate to $NaCl_2$ solution	continuous production.
S. cerevisiae (+ enzymes)	calcium alginate	23 days
S. cerevisiae (cane molasses)	calcium alginate	90 days
Z. mobilis	K-carrageenan	800 h
S. cerevisiae	pectin gel horizontal reactor 40 g ethanol/ L.h^{-1}	15 to 30 days,
Pachysolen tannophilus	calcium alginate	26 days, 28-35g/L ethanol
S. carlsbergensis	porous glass+glutaraldehyde	continuous production
S. cerevisiae	alumina beds reactors, 40g/L.h^{-1}	3 months, 2 horizontal

REFERENCES

1. Azhar, A.; Hamdy, M. K. Alcohol fermentation of sweet potato. I. Acid hydrolysis and factors involved. *Biotech. and Bioeng.*, 23:879-86, 1981.
2. Chibata, I. Immobilized microbial cells with polyacrylamide gel and carrageenan and their industrial applications. *In*: Venkatsubramanian, K. *Immobilized microbial cells.* ACS Symposium series 106. 1979, p.187-202.
3. Chibata, I.; Tosa, T.; Fujimura, M. Immobilized living microbial cells. *Ann. Rep. Ferm. Proc.*, 6:1-22, 1983.
4. Chibata, I.; Tosa, T.; Sato, T. Immobilized biocatalyst to produce amino acids and other organic compounds. *In*: Laskin, A.I. *Enzyme and immobilized cells in biotechnology.* The Benjamin/ Cummings, U.S.A. 1985, p.37-70.
5. Chibata, I.; Tosa, T.; Sato, T. Methods of cell immobilization. *In*: Demain, A. L.; Solomon, N. A. *Manual of industrial microbiology and biotechnology.*, 1986, p. 217-29.
6. Daugulis, A. J.; Krug, T. A.; Choma, C. E. T. Filament formation and ethanol production by *Zymomonas mobilis* in adsorbed cell bio reactor. *Biotech. and Bioeng.*, 27:626-31, 1985.
7. Durand, G.; Navarro, J. M. Immobilized microbial cells. *Process Bioch.*, 13(9):14-23, 1978.
8. Furusaki, S.; Seki, M. Use and engineering aspects of immobilized cells in biotechnology. *Adv. Biochem. Eng. Biotech.*, 46:162-82, 1992.
9. Hamdy, M. K.; Kim, K.; Rudtke, C. A. Continuous ethanol production by yeast immobilized onto channeled alumina beads. *Biomass*, 21:189-206, 1990.
10. Inloes, D. S.; Taylor, D. P.; Cohen, S. N.; Michaels, A. S.; Robertson, C. R. Ethanol production by *Saccharomyces cerevisiae* immobilized in hollow-fiber membrane bio reactor. *Appl. Environ. Microbiol.*, 46:264-78, 1983.
11. Inlow, D.; McRae, J.; Ben-Bassat, A. Fermentation of corn starch to ethanol with genetically engineered yeast. *Biotech. and Bioeng.*, 32:227-34, 1988.
12. Jain, W. K.; Toran-Diaz, I.; Baratti, J. Continuous production of ethanol from fructose by immobilized growing cells of *Zymomonas mobilis*. *Biotech. and Bioeng.*, 27:61-20, 1985.
13. Kierstan, M.; Bucke, C. The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels. *Biotech. and Bioeng.*, 19:387-97, 1977.
14. Kim, K.; Hamdy, M. K. Acid hydrolysis of jerusalem artichoke for ethanol fermentation. *Biotech. and Bioeng.*, 28:138-41, 1986.
15. Kim, K.; Hamdy, M. K. Depolymerization of starch by high pressure extrusion. *J. Food Sci.*, 52(5):1387-90, 1987.
16. Klein, J.; Wagner, F. Methods for the immobilization of microbial cells. *In*: Chibata, I.; Wingard, L. B. *Appl. biochem. and bioeng.* Academic Press, New York, 1983, p.11-55.
17. Kolot, F. B. New trends in yeast technology-immobilized cells. *Process biochem.*, 15:2-8, 1980.
18. Kumakura, M.; Yoshida, M.; Asano, M. Preparation of immobilized yeast cells with porous substrates. *Proc. Biochem.*, 27:225-9, 1992.
19. Linko, P.; Linko, Y. Y. Applications of immobilized microbial cells. *In*: Chibata, I.; Wingard, L. B. *Immobilized microbial cells.* Academic Press, New York, 1983, p. 53-151.
20. Lother, A. M. Introdução de um sistema de fermentação contínua utilizando células imobilizadas em alumina. Piracicaba, ESALQ/ Departamento de Ciência e Tecnologia Agroindustrial, 1994. 28p.
21. Lother, A. M.; Oetterer, M. Imobilização de células a partir de substrato amiláceo. *In*: *Seminários do Curso de P.G. em Ciência e Tecnologia de Alimentos.* ESALQ/ Departamento de Ciência e Tecnologia Agroindustrial, 1994.
22. Melin, E.; Shieh, W. K. Continuous ethanol production from glucose using *Saccharomyces cerevisiae* immobilized on fluidized micro carriers. *Chem. Eng. J.* 50:B17-B22, 1992.
23. Messing, R. A.; Oppermann, R. A. Pore dimensions for accumulating biomass. I. Microbes that reproduce by fission or by budding. *Biotech. and Bioeng.*, 21:49-58, 1979.
24. Navarro, J. M.; Durand, G. Modification of yeast metabolism by immobilization onto porous glass. *European J. Appl. Microbiol.*, 4:243-54, 1977.
25. Navarro, A.; Marangoni, H.; Plaza, I. M.; Callieri, D. Horizontal reactor for the continuous production of ethanol by yeasts immobilized in pectin. *Biotech. Lett.*, 6:465-70, 1984.
26. Nguyen, V. T.; Shieh, W. K. Continuous ethanol fermentation using immobilized yeast in a fluidized bed reactor. *J. Chem. Tech. Biotechnol.* 55:339-46, 1992.
27. Oetterer, M. Ethanol production by two immobilized cultures using starch as substrate. Pos Doctor Report. CNPq-University of Georgia. 1989-1990, 105p.
28. Oetterer, M.; Hamdy, M. K. Desenvolvimento do processo de imobilização de células para bioconversão de substrato amiláceo. *In*: *Congresso Brasileiro de Ciência e Tecnologia de Alimentos*, 13., São Paulo. *Resumos*. São Paulo, SBCTA, 1992, p.139.
29. Phillips, C. R.; Poon, Y. C. *Immobilization of cells.* Springer/ Verlag, Berlin, 1988, p.167.
30. Pradella, J. G. da C. Estudo da fermentação alcoólica contínua de melaço de cana-de-açúcar com células imobilizadas. São Paulo, 1987. 234p. (Doutorado - Escola Politécnica/USP).
31. Rosevear, A.; Kennedy, J. F.; Cabral, J. M. S. *Immobilized enzymes and cells.* I.O.P. Publishing, England, 1987, p. 248.
32. Sitton, O. C.; Gaddy, J. L. Ethanol production in an immobilized cell reactor. *Biotech. and Bioeng.*, 22:1735-48, 1980.
33. Sliminger, P. J.; Bothast, R. J.; Black, L. T.; McGhee, J. E. Continuous conversion of D-xylose to ethanol by immobilized *Pachysolen tannophilus*. *Biotech. and Bioeng.*, 24:2241-51, 1982.
34. Tampion, J.; Tampion, M. D. *Immobilized cells: principles and applications.* Cambridge University Press, 1988, p.257.
35. Vieth, W. R.; Venkatsubramanian, K. Immobilized microbial cells in complex biocatalyst. pp 1-11. *In*: Venkatsubramanian, K. *Immobilized microbial cells.* ACS Symposium series, 106, Washington, 1978, 257p.
36. Wada, M.; Kato, J.; Chibata, I. Electron microscopic observation on immobilized growing yeast cells. *J. Ferment. Tech.*, 58(4):327-31, 1980.
37. Williams, D.; Munnecke, D. M. The production of ethanol by immobilized yeast cells. *Biotech. and Bioeng.*, 23:1813-25, 1981.

Rev. Microbiol., São Paulo, **26**(3): 160-164, 1995

EFFECTIVENESS OF *BRADYRHIZOBIUM SP* INOCULANTS ON DIFFERENT SUBSTRATES[1]

Márcia do Vale Barreto de Figueiredo[2]
Newton Pereira Stamford[3]
José Julio Vilar[4]
Hélio Almeida Burity[5]

ABSTRACT

The aim of this work was to establish the ideal conditions for survival and effectiveness of *Bradyrhizobium* sp on alternative substrates with varying matric potentials. Diatomite, dried sugarcane vinasse, vermiculite and urban compost were used as substrates and compared to peat. Strain UMKL-58 of *Bradyrhizobium* sp, selected in a previous work, was used as inoculant. Substrates were equilibrated at water matric potentials (Ψm) of -0,33, -1,0 and -3,0 bar and incubated for 240 days. Strain survival was evaluated by the plate dilution technique on days 0, 12, 30, 60, 90, 120, 150, 180 and 240 after substrate inoculation and also by plant infection using the legume cunhã (*Clitoria ternatea* L.). Biological nitrogen fixation as a function of the substrate was also studied. The results demonstrate that *Bradyrhizobium sp* survived better in diatomite with a Ψm of -3,0 bar, although at the other water potentials its behaviour was similar to that observed in peat. For all the substrates, low water content (low matric potentials) reduced survival rates and also affected the quality of the inoculum, decreasing the effectiveness of the strain.

Key words: Nitrogen fixation, nodulation, substrates, matric potentials

INTRODUCTION

Nodulating legumes have two available sources of nitrogen: mineral nitrogen and/or fertilizers in soil, and atmospheric nitrogen (dinitrogen), which is biologically fixed by symbiotic bacteria of the genera *Rhizobium* or *Bradyrhizobium*.

One way of placing these bacteria very close to the plant roots is by inoculation of the seeds. However, when developing appropriate inoculants, a problem arises from the scarcity of materials suitable as vehicles. The majority of inoculants available in the market use peat as substrate for bacterial growth, owing to its high organic matter content, high water-retention capacity and ready availability (Roughley and Pulsford, 1982). These characteristics provide rapid proliferation and higher longevity to the bacteria introduced, allowing their survival for several months at a high population density (Alvarez *et al*, 1970).

The survival of bacteria on a substrate depends on its physical, chemical and biological characteristics, as well as the efficiency of the strain employed. The effects of substrate water

[1] Projeto financiado pela Secretaria de Ciência e Tecnologia e CNPq.
[2] Bióloga. Msc. Lab. de Microbiologia do Solo. Empresa de Pesquisa Agropecuária do Estado de Alagoas - EPEAL.
[3] Eng. Agron. Dr. Sc. Prof. Titular, Deptº de Agron. UFRPE.
[4] Eng. Agron. PhD. Prof. Adjunto. Deptº de Agron. UFRPE.
[5] Eng. Agron. PhD. Empresa Pernambucana de Pesquisa Agropecuária-IPA/EMBRAPA.

content on survival should be considered separately for sterilized and non-sterilized inoculants, as there is an obvious interaction between rhizobia and contaminants at specific humidity levels. The water content of a culture has an effect on the quantities of rhizobia, since not only the initial level is critical but there is also a well-defined relationship between death rate and the rate of water loss during storage of the inoculant (Roughley and Pulsford, 1982). During the preparation of the inoculant it is essential for the water content to be appropriate, so as to be able to determine a specific vehicle. The ideal humidity limit for an inoculant should take into consideration bacterial growth, the peak population reached after mixing and bacterium survival.

The aim of the present investigation was to determine the ideal humidity conditions for survival of *Bradyrhizobium* on different substrates, and, additionally, to study the effectiveness of the strain in relation to durability of the inoculant.

MATERIALS AND METHODS

The substrates used were: Diatomite (Carro Quebrado-Rio Grande do Norte state, Brazil); Urban compost (Sanitary landfill of Recife's waste processing plant, Curado, Pernambuco state, Brazil); Dried vinasse (Estreliana sugar mill, Ribeirão Preto, Pernambuco state, Brazil); Expanded vermiculite (Eucatex S/A, São Paulo, Brazil) and Peat (CNPA - Km 47, Rio de Janeiro, Brazil).

The substrates were air dried and sieved through a 200 mesh (0.074 mm) sieve. Physical analysis of the materials was then carried out according to the methodology prescribed by EMBRAPA (EMBRAPA, 1979), followed by chemical analysis according to the norms set by the Ministry of Agriculture (Brazil, 1983). The results are shown in TABLE 1.

The pH of the different materials was corrected to 6.8-7.0 by addition of calcium carbonate to acid substrates and citric acid to alkaline substrates.

A uniform volume of liquid was added to all the substrates, the standard being based on the calculations of lowest matric potential (-3.0 bar) specified for urban compost. Further additions of liquid to reach different water potentials were carried out according to the water retention curve of each material, so as to provide the same matric potentials for the various substrates.

Samples of 40g of material per pack of inoculant were placed in a polypropylene (0.05 mm thick) bag; the appropriate quantity of cells suspended in YEM medium was aseptically injected using a sterile hypodermic syringe. The area around the perforation was disinfected with alcohol immediately after inoculation and then sealed with sterile adhesive tape (Roughley, 1970 and Date, 1976). The bags were then stored at 5°C.

Plate counts were carried out according to Vincent (1970) on days 0, 12, 30, 60, 90, 120, 150, 180 and 240 after inoculum preparation, using cycloheximide to prevent fungal contamination.

Plant dilution counts were carried out (Vincent, 1970) from day 30 to day 240 in a growth chamber using cunhã (*Clitoria ternatea* L.) as test plant, the seeds having been scarified with concentrated sulfuric acid (Zarough and Munns, 1980). The effectiveness of the inoculants was evaluated in

TABLE 1. Physical analysis of substrates and respective matric potentials (ψm)

Substrate	ψm (bar)								Physical analysis		
	-0.10	-0.33	-0.50	-1.00	-3.00	-5.00	-10.00	-15.00	Ur+	dp++	da++++
	Water content g/g								g/cm3		
Peat	1.91	1.08	0.82	0.76	0.67	0.65	0.62	0.57	0.16	1.49	0.49
Diatomite	1.61	1.50	1.40	1.15	0.68	0.41	0.35	0.31	0.07	1.80	0.32
Vermiculite	1.20	1.09	0.82	0.55	0.35	0.27	0.23	0.18	0.07	2.23	0.59
Dried vinasse	0.73	0.60	0.51	0.43	0.28	0.25	0.23	0.21	0.06	2.07	0.91
Urban compost	0.58	0.54	0.41	0.35	0.24	0.18	0.16	0.15	0.04	2.26	1.11

Ur+ = residual humidity
dp++ = particle density
da+++ = apparent density

Leonard jars after 240 days.

The experimental design was completely randomized block designed with four repetitions, and the treatments with three factors (time, matric potential and substrates) with two repetitions and eight sub-samplings. Correlation coefficients were calculated between the plant dilution count (MPN) and plate count methods.

RESULTS AND DISCUSSION

FIGURE 1 shows the effect of water matric potentials on the logarithm of the number of viable cells (NVC) in relation to the substrate. Irrespective of the level of matric potential, peat and diatomite show values of NVC statistically greater than those recorded for the other substrates. The best level of matric potential occurred at -1.00 bar, except for diatomite at -3.00 bar where NVC was greatest. It is possible that the potentiality of the diatomite, when studied at its best level of matric potential, is greater than that of peat. The low values of NVC at -0.33 bar may be due to the fact that the greater water content displayed by this potential inhibits to some extent the gaseous exchange needed for aerobic microorganisms, in keeping with results obtained by Thompson (1980) and Date (1976).

FIGURE 2 shows the survival of bacteria on the five substrates over a 240 days period after mixing of the inoculum, with significant interaction. The first count at t=0 established the bacteria absorption capacity for each substrate on the basis

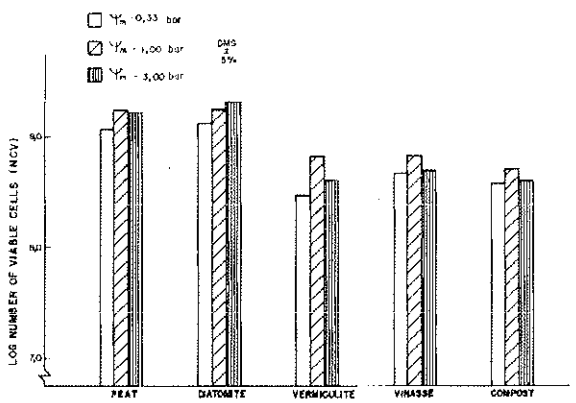


FIGURE 1. Effect of matric potential on the survival of *Brodyrhizobium* sp. in different alternative substrates.

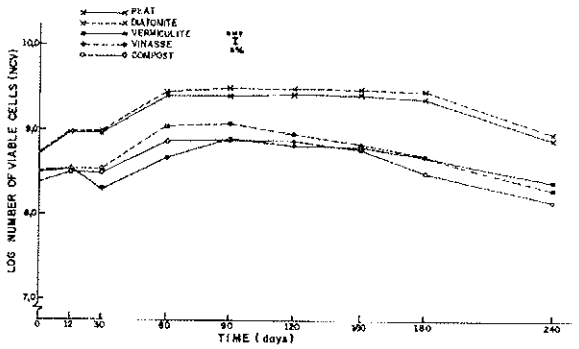


FIGURE 2. Effect of time on the survival of *Brodyrhizobium* sp. in different alternative substrates, as a function of matsic potential.

of recuperation, evaluated by the number of microorganisms obtained per count of the inoculant and the number of cells added per gram of inoculant. The greatest number of bacteria for all the substrates were recorded 90 days after incubation at 5°C, this being considered the peak of growth. The results obtained are in agreement with those reported by Date (1976) on the survival of *Rhizobium meliloti* on sterile peat under similar storage conditions.

A comparison of estimates of the quantity of inoculant can be performed on the basis of plate counts, where the estimates are compared by both readings in the same dilution series. The difference between the two estimates was within the 55% reliability (0.82) of the plant infection method. The correlation coefficient (r = 0.971) indicated a high significance between MPN and plates for the 30 days storage period.

In general, the counts by plant infection underestimated the quantity of viable bacteria when compared to the plate counts. Similar results have been found by Date and Vincent (1962), Hiltbold et al (1986) and Kremer and Peterson (1982), who confirmed a lower count by the former method in relation to the latter. Brockwell (1963) studied the precision of the MPN method and the estimates oscillated between 89 and 131% of those obtained with the plate count method.

On evaluating the bacterial population of the vehicles by the dilution and plant infection and plate count methods after storage for 240 days, a significant correlation coefficient (r = 0.900) was observed. With respect to the experiments were varying m of the substrates were tested (TABLES 2 and 3), it can be seen that values of -1.00 and -3.00 bar did not

differ statistically concerning the number and dry matter of nodules and nitrogenase activity. There was a significant interaction between shoot dry matter and total N accumulated. However, at a m of -0.3 bar, lower values were observed for all the parameters studied.

The data obtained showed that greater m values affected the quality of the inoculum, reducing the effectiveness of the strain and probably altering the bacterial metabolic activity, with influence on the site of infection. These results are in agreement with Hamdi and Worrall *et al*, cited by Bergersen (1982), who state that humidity affects the movement of bacteria and alters the infection rate, reducing nodulating activity and energy efficiency.

TABLE 2. Nodulation and nitrogenase activity of cunhã *(Clitoria ternatea* L.) as a function of substrate matric potential (ψm).

Substrate	Nodulation		Nitrogenase activity
	Number	Weight	
	n°/pot	g/pot	nmol C2H4/plant.h-1
Peat	23.0a	0.21a	25.50ab
Diatomite	24.0a	0.21a	33.15a
Vermiculite	16.0 bc	0.17ab	16.30 bc
Dried Vinasse	13.0 c	0.12 b	15.58 bc
Urban compost	16.0a	0.19a	23.97abc
Matric potencial			
ψm -3.00	21.0a	0.18a	23.04a
ψm -1.00	22.0a	0.18a	24.22a
ψm -0.33	17.0 b	0.14 b	17.22 b
CV(%)	11.66	33.21	38.06

In each column, the means followed by the same letter do not differ statistically from each other at the level of 5% probability,according to Tukey's test.

CONCLUSIONS

a) Strain UMKL-58 of *Bradyrhizobium* sp survived better in the diatomite and peat substrates. However, for all the vehicles tested, the growth and survival of these bacteria at high Ψm were reduced as a consequence of greater water content.

b) The different vehicles varied in their capacity to retain water. Therefore, water potential represented by Ψm is a more precise parameter than the gravimetric water content, given that movement, absorption, evaporation and retention are functions of the energy of the water in the substrate.

c) The quality of the inoculum was affected by greater Ψm that reduced strain effectiveness, probably due to changes in metabolic activity that influenced the site of infection.

RESUMO

Efetividade do inoculante com *Bradyrhizobium* sp em diferentes substratos

Objetivando determinar as condições ideais para a sobrevivência e efetividade do

TABLE 3. Production of dry matter and total nitrogen accumulated in cunhã (*Clitoria ternatea* L.) as a function of ψm in the various substrates

Substrate→	Peat		Diatomite		Vermiculite		Dried Vinasse		Urban Compost	
ψm ↓	MS	Total-N	MS	Total-N	MS	Total-N	MS	Total-N	MS	Total-N
g/pot	mg/pot	g/pot	mg/pot	g/pot	mg/pot	g/pot	mg/pot	g/pot	mg/pot	
-3.00	2.92a	90.83a	3.09a	90.02a	2.41 b	66.52 b	2.34 b	64.25 b	2.17 b	56.21 b
-1.00	3.00a	94.60a	2.81 b	83.61 b	2.73a	82.59a	2.66a	77.87a	2.39a	65.77a
-0.33	2.60 b	71.57 b	2.54 c	72.71 c	2.20 c	53.33 c	2.12 c	52.59 c	1.90 c	46.13 c
Control										
TN	2.93	93.92								
TA	0.57	5.49								
CV (%)	4.62	5.83								

In each column, the means followed by the same letter do not differ statistically from each other at the level of 5% probability, according to Tukey's test.

Bradyrhizobium sp em substratos alternativos com diferentes potenciais matriciais, foi conduzido um experimento utilizando-se como veículo de inoculação os substratos: diatomita, vinhaça seca, vermiculita e composto urbano em relação à turfa. A inoculação foi procedida com a estirpe UMKL-58, selecionada em trabalhos anteriores. Os substratos foram submetidos aos Ψm de -0,33, -1,0 e -3,00 bar e incubados por 240 dias. Para a qualificação dos veículos foram feitos testes de sobrevivência da estirpe nos materiais utilizando-se os métodos de diluição em placas e de infecção em plantas com a leguminosa cunhã (*Clitoria ternatea* L.) nos intervalos de 0, 12, 30, 60, 90, 120, 150, 180 e 240 dias após a inoculação, como também, avaliação da eficiência da fixação biológica do N_2 em função do veículo de inoculação. Os resultados demonstraram que o *Bradyrhizobium* sobreviveu melhor na diatomita submetida a Ψm -3,0 bar e nos demais potenciais foi comparável a turfa. Para todos os veículos, observou-se que altos valores de Ψm reduziram a taxa de sobrevivência da bactéria como também afetaram a qualidade do inóculo diminuindo a efetividade da estirpe.

Palavras-chave: fixação de nitrogênio, nodulação, substrato potencial matricial.

REFERENCES

1. Alvarez, A., Mas, J. and Margosion, C. Producción industrial de inoculantes. *In*: REUNIÃO LATINO AMERICANA DE *Rhizobium*, 1970, Rio de Janeiro. *Anais*. Rio de Janeiro: Instituto de Pesquisas Agropecuárias do Centro Sul, 1970, p. 346-348.
2. Brasil. Ministério da Agricultura. Laboratório Nacional de Referência Vegetal. *Análise de Corretivos, fertilizantes e inoculantes, métodos oficiais* [s.l.:s.n.], 1983, 103 p.
3. Bergerson, F.J. *Root Nodules of legumes*: Structure and Functions. New York: Wiley Research Studies Press, 1982, p. 164.
4. Brockwell, J. Accuracy of plant injection technique for counting population of *Rhizobium trifolii*. *Applied Microbiology*: Washington, v. 11, 1963, p. 377-383.
5. Date, R.A. Legume inoculant production. *Proceedings of the Indian. National Science Academy*, Section B, Bangalone, v. 40, n. 6, 1976, p. 667-686.
6. Date, R.A. and Vincent, J.M. Determination of the number of root nodule bacteria in the presence of other organismis. *Australian Journal Experimental Agriculture and Animal Husbandry*, Victoria, v. 2, p. 5-7, 1962.
7. EMBRAPA. Serviço Nacional de Levantamento e Conservação de Solos. *Manual de Análises do Solo*. Rio de Janeiro, v. 1, 1979.
8. Hiltbold, D.A.E., Thurlow, D.L. and Skipper, H.D. Evaluation of commercial soybean inoculants by various techniques. *Agronomy Journal*, Madison, v. 72, 1980, p. 675-681.
9. Kremer, R. J. and Peterson, H. L. Effect of inoculant carrier on survival of *Rhizobium* on inoculated seeds. *Soil Science*, Baltimore, v. 134, n. 2, 1982, p. 117-125.
10. Roughley, R.J. The preparation and use of legume seed inoculants. *Plant Soil*, v. 32, 1970, p.675-701.
11. Roughley, R.J. and Pulsford, D.J. Production and control of legume inoculants. *In*: Vincent, J.M. *Nitrogen Fixation in Legume*. Austrália: Academic Press, 1982, p. 193-209.
12. Thompson, J.A. Production and quality control of legume inoculants. *In*: Bergersen, F.J. *Methods for evaluation biological nitrogen fixation*. New York: John Wiley, 1980. p.490-533.
13. Vincent, J.M. *A manual for the pratical study of Rhizobium of root nodule bacteria*. Oxford: Blackwells Scientific Publication, 1970, 164 p.
14. Zaroug, M.G. and Munns, D.N. Screening strains of *Rhizobium* for the tropical legumes *Clitoria ternatea* and *Vigna trilobata* in soil of different pH. *Tropical Grasslands*, v. 14, n. 1, 1980, p. 28-33.

Rev. Microbiol., São Paulo, **26**(3): 165-168, 1995

EFFECT OF YEAST EXTRACT AND MEDIUM PH ON POLYGALACTURONASE PRODUCTION BY *PENICILLIUM EXPANSUM*

Mara Lúcia Albuquerque Geöczc
Jorge Luiz Cavalcante Coelho
Elza Fernandes de Araújo
Daison Olzany Silva[1]

SHORT COMMUNICATION

ABSTRACT

Polygalacturonase activity of *Penicillium expansum* was evaluated in the culture filtrate after removal of the mycelial mass. The best culture condition for enzyme production was growth in unbuffered mineral medium in the absence of yeast extract. Production of the enzyme was dependent on growth medium pH and was maximal at acid pH.

Key words: Polygalacturonase, *Penicillium expansum*, pH, Yeast extract

The polygalacturonases (PG) produced by fungi are very useful for industrial application in the processing of raw plant material. These enzymes have long been used by the food industry for juice and wine clarification (7, 11). The technological application of PG for treatment of natural fibers such as ramie and linen is being currently developed in order to facilitate fiber degumming, and also to prevent pollution and economic problems (1). The production of pectinases by microorganisms is influenced by growth medium composition and culture conditions such as pH, temperature, aeration, ionic strength, and inoculum concentration (5, 6). Some investigators have concluded that amino acids repress PG synthesis (4, 8, 12). The aim of the present investigation was to study the effect of yeast extract and of culture medium pH on polygalacturonase production by *Penicillium expansum*.

Sporulated *Penicillium expansum* cultures were maintained on slanted agar-oatmeal medium (15 g and 40 g, respectively, in 1000 mL distilled water) stored in a refrigerator at 5-10°C. For inoculum preparation, one of the stored cultures was inoculated into test tubes containing the same medium and incubated at 25°C for 5 days. The spores were inoculated into the growth medium at a concentration of 2.8×10^6 spores mL^{-1}. The culture medium for fungal growth and PG production consisted of mineral medium (MM) (3) containing 0.3% citrus pectin (Eskisa) as a carbon source and as an enzyme inducer. The following media were used to study the effect of pH on PG production: 1) unbuffered MM consisting of 2.0 g KH_2PO_4, 0.62 g K_2HPO_4, 1.1 g $MgSO_4.7H_2O$ and 1.0 g $(NH_4)_2SO_4$ in 1000 mL distilled water, pH 6.3; 2) buffered MM consisting of the same unbuffered MM medium except for K_2HPO_4 at 7.0 g/L and pH 7.0. The effect of yeast extract on PG production was evaluated by adding 0.06% yeast extract to the

[1] To whom correspondence should be sent, at the address: Departamento de Microbiologia, Universidade Federal de Viçosa, 36571-000, Viçosa, MG. Telephone: (+55-31) 899-2553, FAX: (+55-31) 899-2573

culture medium. Cultures were grown with shaking (150 rpm) at 25°C. Fungal growth was monitored on the basis of mycelial dry weight. Enzyme activity and protein content of the culture filtrate were determined over a period of 120 hours of incubation. Mycelium was separated from the culture medium with a 400 mesh sieve (37µm pores), washed in distilled water and dried to a constant weight in an oven at 105°C. PG activity was determined in a reaction mixture consisting of 1 mL of culture supernatant, 3 mL of 0.3% polygalacturonic acid in 50 mM sodium acetate buffer, pH 4.5, and 0.1 M NaCl, for 20 min at 40°C. The reducing sugars released were measured by the 3,5 dinitrosalicylic acid method of Miller (10) using galacturonic acid as standard. Protein was measured in the culture filtrates by the Bradford method (2) using bovine serum albumin as standard. One unit of PG (PG units) was defined as µmoles of galacturonic acid released per minute (U) per µg of protein (PG Specific Activity) or µmoles of galacturonic acid released per minute (U) per mililitre (PG Activity).

FIGURE 1 (A and B) shows that there was a fall in PG production when the culture medium was supplemented with the yeast extract, reaching maximal enzyme activity and mycelial mass production after 36 to 48 hours of culture. In contrast, the fungus grown in culture medium not supplemented with yeast extract presented high PG production, although biomass production was similar to that obtained with the supplemented medium. The highest enzyme activity was obtained after 24 to 48 hours of culture. Maximal mycelial production was obtained 48 hours after inoculation.

A fall in enzyme specific activity was observed after 48 hours of growth in both culture conditions (FIGURE 1B). However, the total enzyme activity remained stable during all the growth period, indicating that the PG produced is quite stable (FIGURE 1A).

With respect to the mechanism by which yeast extract leads to a decrease in PG production by *P. expansum*, some investigators have suggested that the amino acids present in the extract may be repressing PG synthesis (8). Furthemore, Spalding *et al.* (12) demonstrated PG repression in *P. expansum* when methionine or gentamic acid were combined with pectin and sodium pectate.

FIGURE 1C shows the variation in culture medium pH during *P. expansum* growth. There was

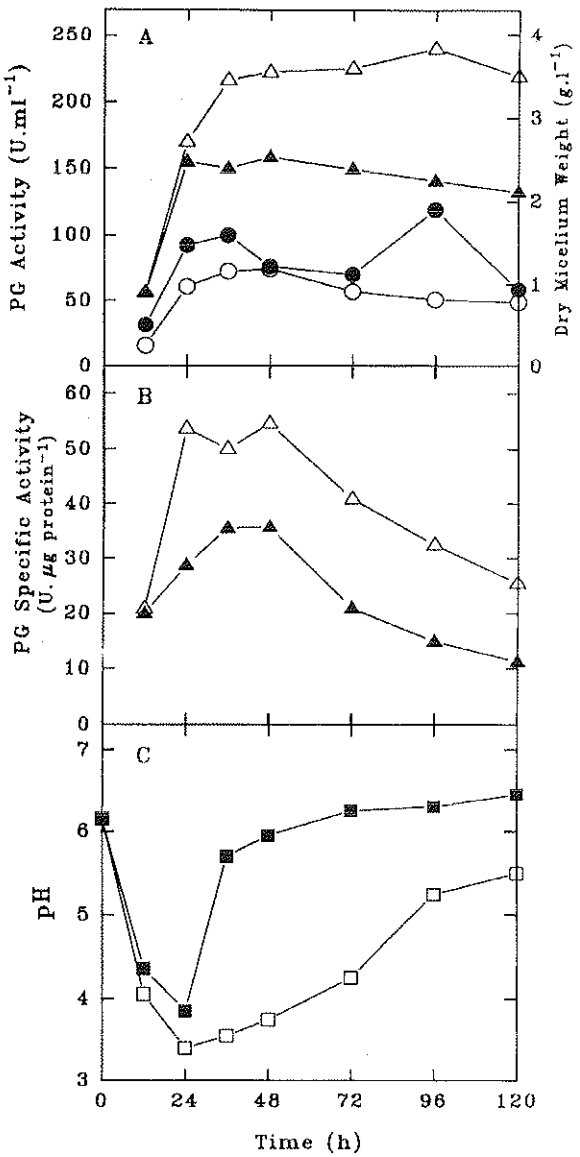


FIGURE 1. PG activity (A), specific PG activity (B) and pH variation (C) during the growth of *P. expansum* cultured in unbuffered mineral medium plus citrus pectin (Eskisa), with no yeast extract (△ □) and with 0.06% yeast extract (▲ ■) at 25°C and 150 rpm. Mycelial dry weight of the fungus grown in the absence (○) or presence (●) of 0.06% yeast extract.

a marked variation pH values under both culture conditions. In the presence of yeast extract, the initial pH was reduced to 3.9 after 24 hours and increased to 6.3 after 120 hours of incubation. In the absence of yeast extract, the initial pH decreased to 3.4 after 24 hours of incubation, also increasing

thereafter, although more slowly than in the presence of yeast extract. The results suggest an effect of the yeast extract on *P. expansum* metabolism, with acidification of the culture medium occurring in its absence. This situation seems to favor the synthesis and/or secretion of the enzyme. From an industrial viewpoint, this is an advantage because there is no need to control the pH of the culture medium, an also because of the reduced risk of contamination by other microorganisms (9, 13).

Leuchtenberger *et al.* (8) reported that the accumulation of acid metabolites demonstrated by the rapid decrease in PG values in simple culture medium (mineral medium containing pectin and glucose as carbon source) favored the production of PG by *Aspergillus niger*, since the activity of the enzyme was detected earlier in the culture filtrates.

FIGURE 2 shows that when buffered culture medium at pH close to 7.0 was used, total PG activity and specific PG activity were similar and remained constant over the whole incubation period, both in the presence or absence of yeast extract.

These results indicate that the presence of yeast extract did not affect PG production by *P. expansum*. More importantly, a culture medium pH close to 7.0 had a negative effect on enzyme production when comparing the total and specific PG activity values obtained in this experiment (approximately 170 $U.mL^{-1}$ and 12 $U.\mu g\ protein^{-1}.min^{-1}$, respectively) with the values recorded in the previous experiment (approximately 230 $U.mL^{-1}$ and 55 $U.\mu g\ protein^{-1}.min^{-1}$, respectively). The data reinforce the hypothesis that PG production by *P. expansum* is dependent on growth medium pH and is maximal at acid pH. We suggest that the yeast extract may act by preventing acidification of the culture medium, thus decreasing PG synthesis and/or secretion. Similarly, Maldonado *et al.* (9) noted that the pH of the growth medium used for *Aspergillus* sp decreased to 2.7 after 60 hours of culture, coinciding with the most productive phase in the culture.

The present results indicate that unbuffered culture medium should be used for PG production by *Penicillium expansum*, with obvious economy in the production of the enzyme for industrial use. The data also suggest that repression of PG synthesis should not be attributed to the amino acids present in the yeast extract (0.06%) but rather to the change in physiological behavior of the fungus in the presence of the yeast extract, with induction of PG synthesis at a lower pH.

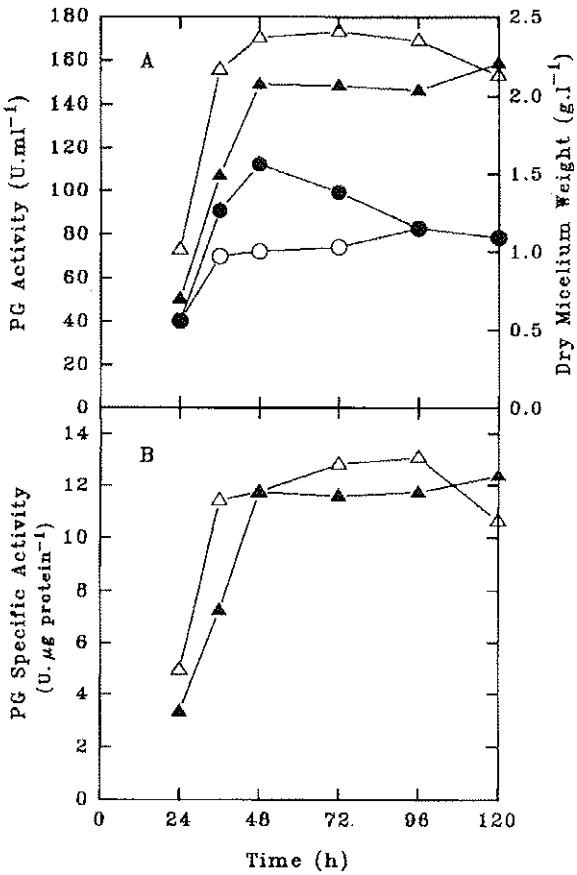


FIGURE 2. PG activity (A) and specific PG activity (B) of *P. expansum* cultured in buffered MM plus 0.3% citrus pectin (Eskisa), with no yeast extract (Δ) and with 0.06% yeast extract (▲) at 25°C and 150 rpm. Mycelial dry weight of the fungus grown in the absence (○) or presence (●) of 0.06% yeast extract.

RESUMO

Efeito de extrato de levedura e pH do meio de cultura na produção de poligalacturonase por *Penicillium expansum*

A atividade de poligalacturonase extracelular de *Penicillium expansum* foi dosada nos filtrados das culturas, após a remoção da massa micelial, a qual foi usada na determinação do crescimento do microrganismo. As melhores condições de crescimento para produção de enzima, foram o uso de

meio mineral não tamponado e ausência de extrato de levedura. A produção da enzima foi dependente do pH do meio de crescimento, sendo máxima em pH ácido.

Palavras-chaves: Poligalacturonase, *Penicillium expansum*, pH, extrato de levedura

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from the Brazilian Agencies CNPq and FINEP.

REFERENCES

1. Baracat, M.C.; Vanetti, M.C.D.; Araújo, E.F.; Silva, D.O. - Growth conditions of a pectinolytic *Aspergillus fumigatus* for degumming of natural fibres. *Biotechnol. Lett.*, 13: 693-696, 1991.
2. Bradford, M.M. - Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.*, 72: 248-254, 1976.
3. Brumano, M.H.N.; Coelho, J.L.C.; Araújo, E.F.; Silva, D.O. - Production of pectin lyase by *Penicillium griseoroseum* as a function of the inoculum and culture conditions. *World J. of Microbiol. and Biotechnol*, 9: 225-228, 1993.
4. Chopra, S.; Mehta, P. - Influence of various nitrogen and carbon sources on the production of pectolytic, cellulolytic and proteolytic enzymes by *Aspergillus niger*. *Folia Microbiol.*, 30: 117-125, 1985.
5. El-Refai, A.A.; Metwalli, S.M.; El-Sebaiy, L.A. - Influence of pH, inoculum concentration, aeration and growth period on production of pectolytic enzymes by *Aspergillus awamori* 16. *Chemische Mikrobiologie und Technologie der Lebensmittel*, 115-117, 1984.
6. Eveleigh, D.E.; Montenecourt, B.S. - Increasing yields of extracellular enzymes. - *In*: Perlman, D., ed. - Advances in Applied Microbiology. Academic Press, v. 25, 1979, p. 57-74.
7. Ghildyal, N.P.; Ramakrishna, S.V.; Devi, P.N.; Lonsane, B.K.; Asthana, H.N. - Large scale production of pectolytic enzyme by solid state fermentation. *J. Food Sci. Technol.*, 18: 248-251, 1981.
8. Leuchtenberger, A.; Friese, E.; Ruttloff, H. - Synthesis of variable enzyme spectrum by immobilized mycelium of *Aspergillus niger*. *Zentralbl. Mikrobiol.*, 144: 139-149, 1989.
9. Maldonado, M. C.; Navarro, A.; Callieri, D.A. - Production of pectinases by *Aspergillus* sp using differently pretreated lemon peel as the carbon source. *Biotechnol. Lett.*, 8: 501-504, 1986.
10. Miller, G.L. - Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analyt. Chem.*, 31: 426-431, 1959.
11. Rombouts, F.M.; Pilnik, W. - Pectinases and other cell-wall degrading enzymes of industrial importance. *Symbiosis*, 2: 79-90, 1986.
12. Spalding, D.H.; Wells, J.M.; Allison, D. W. - Catabolite repression of polygalacturonase, pectin lyase and cellulase synthesis in *Penicillium expansum*. *Phytopathology*, 63: 840-844, 1973.
13. Zetelaki-Horváth, K. - Possibilities for control of pectolytic enzyme formation by *Aspergillus niger* in continous culture. *Acta Alimentaria*, 12: 12-19, 1983.

Rev. Microbiol., São Paulo, **26**(3): 169-179, 1995

GENETIC STABILITY, CONJUGAL TRANSFER AND EXPRESSION OF HETEROLOGOUS DNA INSERTED INTO DIFFERENT PLASMIDS AND THE GENOME OF *PSEUDOMONAS FLUORESCENS* IN SOIL

Eric Smit
Anneke Wolters
Jan Dick van Elsas

ABSTRACT

The influence of the location of heterologous DNA in *Pseudomonas fluorescens* R2f on gene stability, expression and transfer following introduction into soil was studied. Three strains with markers on different genetic elements were used, i.e a selftransmissible plasmid (RP4p), a mobilizable plasmid (pSKTG) and a chromosomally inserted marker gene cassette (KTG). *In vitro* filter mating experiments showed that the selftransmissible plasmid was transferred with high frequencies (about 10^{-2}) and that this plasmid could mobilize pSKTG with similar frequencies. The chromosomally inserted marker cassette could be mobilized by RP4p to a recipient strain with low frequency (10^{-8}). In sterile soil, transfer of the chromosome could not be detected in the presence of RP4p. When the 3 strains were introduced into 2 soils of different texture planted with wheat, they showed poor survival in Ede loamy sand and good survival in Flevo silt loam. Moreover, a partial, but significant loss of expression of the gentamycin resistance gene *aad*B was observed in Ede loamy sand, but not in Flevo silt loam.

RP4p was found to be transferred from an introduced donor to indigenous bacteria in both non-sterile soils planted with wheat, whereas transconjugants harbouring the mobilizable plasmid pSKTG or the chromosomally inserted marker cassette were not detected when a donor strain lacking an IncP plasmid was used.

INTRODUCTION

The deliberate or accidental release of genetically engineered microorganisms (GEMs) into the environment has led to an increase in gene transfer studies in different environments (4, 17, 18, 23, 40). Gene transfer by conjugation is an important process in an environment like soil (34, 35, 36, 22, 24, 25, 28, 32, 41). Most research has focused on the transfer of selftransmissible plasmids (35, 36, 22, 24, 25, 28, 32, 41), whereas conjugal transfer and genetic stability of non-selftransmissible plasmids (10) and chromosomal insertions have rarely been evaluated in environmental studies. Krasovsky and Stotzky (16) detected conjugal transfer of chromosomal DNA by introducing *Escherichia coli* Hfr donor strains together with *E. coli* recipients into soil microcosms. However, Hfr strains exhibit high mobilization frequencies and are unlikely to be used as GEMs for release.

Besides transfer of recombinant DNA, more attention should be given to the environmental stability and expression of recombinant DNA on non-selftransmissible plasmids or the chromosome (29). The mobilizable IncQ plasmid pSKTG (30), carrying RSF1010 replication and mobilization (*mob*) functions and a marker gene cassette, was constructed to serve as a non-selftransmissible plasmid. Moreover, the marker gene cassette, which contained the neomycin/kanamycin phosphotransferase gene *npt*II and the

aminoglycoside adenylating gene *aad*B, conferring resistance to neomycin/kanamycin and gentamycin respectively, and part of *cry*IVB (30) as a molecular marker, was also inserted into the chromosome.

The present work focused on the stability, expression and possible transfer of chromosomally located heterologous DNA in a *P. fluorescens* strain introduced into soil. Both the fate of the DNA and of the strain was studied for 28 days in one kg microcosms planted with wheat. Chromosomal DNA can potentially be mobilized by IncP plasmids (1, 21, 27); *in vitro* studies have provided extensive data on chromosome mobilization by such plasmids, with frequencies ranging from 10^{-3} to 10^{-9} (9). IncP or other mobilizing plasmids might be naturally present in soil bacteria and be responsible for the dissemination of chromosomally inserted genes (13, 19). Gene stability and expression was also investigated, since these parameters have hardly been studied in soil. Both soil type and the presence of plant roots might influence the stability and expression of genes in soil.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage and growth conditions

Strains of the soil isolate *Pseudomonas fluorescens* R2f (35) harbouring either different plasmids or a chromosomally inserted marker gene cassette, were used in this study (see TABLE 1). The use of plasmid RP4p, RP4 marked with a *Solanum tuberosum* cv *bintje* sequence, *pat*, has been described before (28). Plasmid pSKTG was constructed using RSF1010 sequences (replication functions and the *mob* site) and a gene cassette composed of *npt*II and *aad*B and part of *cry*IVB, coding for a *Bacillus thuringiensis* endotoxin, as a molecular marker (30). The same marker cassette was also inserted into the chromosome of *P. fluorescens* R2fR using the disarmed transposon delivery system of Herrero *et al.* (1990). This system consists of the transposase (*tnp*) gene and 19 bp of the inverted repeats of Tn*5* cloned into a mobilizable suicide vector (pUT). The marker cassette was cloned into a helper plasmid (p18Sfi), allowing it to be excised again using *Sfi*I. The marker cassette was cloned into the *Sfi*I site in the pUT plasmid (yielding pUT/KTG) and transformed into *E. coli* SM10 lambda *pir* (11, 30). Transfer of pUT/KTG to *P. fluorescens* R2fR resulted in *P. fluorescens* R2fmc, resistant to rifampcin (Rp), kanamycin (Km) and gentamycin (Gm). This strain was checked by probing with *cry*IVB and *tnp* (11), followed by Southern blotting of total genomic DNA. Furthermore, tests were done on the stability of the inserted genes by culturing this strain under non-selective conditions and comparing selective and non-selective plate counts (30).

Phage ΦR2f, obtained from agricultural drainage water, was used for donor counterselection because of its specificity for *P. fluorescens* R2f, it was propagated and maintained as described before (28).

All strains were cultured in LB (10 g Difco tryptone; 5 g Difco yeast extract; 5 g NaCl; 1 l H_2O; pH 7.2) with 50 mg/L of the appropriate antibiotics.

TABLE 1. Strains used in this study

Strain	Plasmid markers (resistance)	chr.markers (resistance)
Pseudomonas fluorescens R2f	-	-
P. fluorescens R2fR	-	Rp
P. fluorescens R2fSN	-	Sm Nx
P. fluorescens R2fmc	-	Rp Km Gm
P. fluorescens R2fmc (RP4p)	Ap Tc	Rp Km Gm
P. fluorescens R2fR (pSKTG)	Km Gm	Rp
P. fluorescens R2fR (RP4p)	Ap Tc	Rp

chr. marker = chromosomal marker

Ap: Ampicillin, Tc: Tetracycline, Rp: Rifampicin, Sm: Streptomycin, Nx: Nalidixic acid, Km: Kanamycin, Gm: Gentamycin.

Soils

The two soils used, Ede loamy sand and Flevo silt loam have been described before (25, 35). Portions of freshly collected Ede loamy sand and Flevo silt loam were air-dried to about 10% (Ede loamy sand) or 20% (Flevo silt loam) and sieved (4 mm mesh) before use.

Chromosome mobilization in filter matings

To test the maximal mobilization frequency of the chromosomally inserted marker cassette, filter matings between *P. fluorescens* R2fmc (RP4p) and *P. fluorescens* R2fSN were done (TABLE 2). Aliquots of 50 µL of the washed cell suspensions were pipetted onto the filters, either separately or jointly, and mixed. After 24 h of incubation, filters

TABLE 2. Transfer of RP4p, pSKTG and the chromosomally inserted KTG sequence between *Pseudomonas fluorescens* R2fR donor (D) and *P. fluorescens* R2fSN recipient strains on membrane filters on LB agar plates.

Donor	Transfer frequency per donor of (genetic element):		
	RP4p	pSKTG	KTG
D. (RP4p)	$6\ 10^{-2}$	-	-
D. (pSKTG)	-	[0]	-
D. (RP4p; pSKTG)	10^{-2}	$2\ 10^{-2}$	-
D. (KTG)	-	-	[0]
D. (KTG; RP4p)	$4\ 10^{-2}$	-	$4.5\ 10^{-8}$

- = not performed
[0] = below 10^{-9}

were vortexed in 3 mL LB broth and the resulting suspensions were serially diluted and plated on LB agar containing antibiotics as follows: *P. fluorescens* R2fmc (RP4p) was enumerated on LB with 50 mg/L of Km and 50 mg/L of tetracycline (Tc), *P. fluorescens* R2fSN recipient cells were selected on 100 mg/l streptomycin (Sm) and 100 mg/l nalidixic acid (Nx), and transconjugants were selected on LB agar containing 50 mg/l each of Nx, Sm and Km. Also, donor and recipient were plated on plates containing Sm plus Nx, and Km, to check for mutations. Plates were incubated at 28°C for 4 days before the number of cfu was determined. Transfer frequencies were calculated as transconjugant cfu per donor cfu.

Chromosome mobilization in sterile soil

Mobilization of the chromosomally inserted marker cassette was also studied in sterile soil, using the same donor/recipient combinations as in the filter matings (TABLE 3). The soils, Ede loamy sand and Flevo silt loam were sterilized by irradiation (4 Mrad) prior to use. Portions of 50 g were compressed to a bulk density of 1.4 (wet weight basis) in sterile plastic cups. Washed overnight cultures of donor and recipient strains

TABLE 3: Numbers of donor (D), recipient (R) and transconjugant (T) cfu 7 days after the strains were introduced into sterile Flevo silt loam (FSL) and sterile Ede loamy sand (ELS).

Soil/	Donor	Recipient	Transconjugant cfu	
treatment	cfu	cfu	RP4p	::KTG
FSL, D	$2.3\ 10^{8}$	-	[0]	[0]
FSL, R	-	$1.8\ 10^{8}$	[0]	[0]
FSL, T	$1.9\ 10^{8}$	$0.5\ 10^{8}$	$2.4\ 10^{6}$	[0]
ELS, D	$1.3\ 10^{8}$	-	[0]	[0]
ELS, R	-	$0.9\ 10^{8}$	[0]	[0]
ELS, T	$1.5\ 10^{8}$	$1.1\ 10^{7}$	$2.1\ 10^{3}$	[0]

FSL, D: Flevo silt loam with donor *P. fluorescens* R2fR (*chr*::KTG; RP4p) added at 10^{8} cells per gram; FSL, R: Flevo silt loam with *P. fluorescens* R2fSN (recipient) added at 10^{8} cells per gram soil; FSL, T: Flevo silt loam with both strains added at 10^{8} cells per gram soil. ELS, D: Ede loamy sand with donor added at 10^{8}; ELS, R: Ede loamy sand with recipient added at 10^{8}; ELS, T: Ede loamy sand with both strains added at 10^{8}.

were added to soil to establish population densities of 10^{8} cfu per gram of soil. In order to get a pF value of 2, water was added to the soils to 18% for Ede loamy sand and 38% for Flevo silt loam. Soil portions were then thoroughly mixed using a spatula. Strains were introduced with at least 1 hour intervals to avoid matings in the liquid phase. The microcosms were incubated for 7 days at 20°C in moist chambers to avoid drying, after which they were sampled and processed as described below.

Bacterial survival, genetic stability and transfer in non-sterile soil planted with wheat

Overnight cultures of *P. fluorescens* R2fR (RP4p), *P. fluorescens* R2fR (pSKTG) and *P. fluorescens* R2fmc were transferred to 500 ml LB

to which 50 mg/L of the appropriate antibiotics had been added. After incubation at 28°C at 200 rpm for 16 hours, cells were harvested washed and separately introduced into both soils. Then, demineralized water was added to establish a pF 2 in the soils. Portions of 1 kg of inoculated soil were packed in plant pots (14 cm height, 14 cm diameter) to a bulk density of 1.4 (wet weight) and planted with 1 pregerminated (2 days, 20°C) wheat (*Triticum aestivum cv. sicco)* seedling. To limit evaporation, pots were covered with transparent lids with a small hole to allow the wheat plant to grow through. Pots were incubated in a climate chamber using a cycle of 16 h light and 8 h dark at 20°C and 16°C, respectively. Every other day pots were weighed and water was added up to the original weight.

Determination of expression level of aadB in cells from soil

Since a considerable loss of expression of resistance to Gm of cells of *P. fluorescens* R2fmc extracted from Ede loamy sand was suspected to occur, a separate experiment was done to determine the minimal level of resistance to Gm in cells from soil or starvation culture. *P. fluorescens* R2fmc was added to 50 g portions of Ede loamy sand as described above. The strain was also grown to early log phase, washed three times and suspended in 1/4 Ringers solution (NaCl 2.15 g, KCl 0.075 g, $CaCl_2$ 0.12 g, $NaS_2O_4.7H_2O$ 0.5 g, 1 l H_2O) and incubated in a shaker (100 rpm) at 20°C.

After 10 days in soil or 1/4 Ringers, samples were plated on King's B agar supplemented with 50 mg/L Km plus 0, 10, 20, 30, 40 or 50 mg/L Gm. The number of cfu's was determined and the percentage of the population growing on the plates with different Gm concentrations was calculated and compared to the number on plates containing only Km.

Extraction and enumeration of bacteria from soil

oth sterile and non-sterile systems were sampled as described below. In both systems cells were isolated from soil shortly (three hours) after introduction to determine initial population levels. The sterile systems were further sampled at day 7, while the non-sterile planted systems were sampled at day 10, day 17 and day 28. Plants were gently removed from soil, shaken, and the roots were separated from aboveground parts. Roots plus adhering soil were designated as the rhizosphere sample. The remaining soil and soil from unplanted microcosms was designated bulk soil. Portions of 10 g of both rhizosphere and bulk soil samples were treated as described before (28). To enumerate the introduced strains in non-sterile planted systems, King's B agar (35) was used; cycloheximide (100 mg/L) was added to minimize fungal growth. For *P. fluorescens* R2fR (RP4p) Rp (50 mg/L) and Tc (50 mg/L) were added, for *P. fluorescens* R2fR (pSKTG) and *P. fluorescens* R2fmc Km (50 mg/L) and Gm (50 mg/L) were added in addition to Rp. Furthermore, samples were plated on King's B with either Rp (50 mg/L) or Km (50 mg/L) to assess loss of marker genes or expression.

To obtain indigenous transconjugants, suspensions from the Erlenmeyer flasks were first incubated with equal volumes of lysate of phage ΦR2f to selectively eliminate the donor strain (28). Aliquots of 100µL were then plated on LB agar plates containing 50 mg/L of both Tc and Ap to select for transconjugants harbouring RP4p, or 50 mg/L of both Km and Gm to select for transconjugants harbouring pSKTG or the marker cassette. The total number of culturable bacteria was determined by plating on 0.1 strength TSA (28). Plates were incubated for 2 to 5 days at 28°C.

Analysis of introduced bacteria and transconjugants

Non-selective plates (total counts) and selective plates (with Rp or Km, for the enumeration of introduced bacteria) were randomly used for colony filter hybridization (as described below) to check for the presence of the molecular marker. Colonies (>200 per treatment and sampling) on transconjugant-selective plates (for RP4p, pSKTG and ::KTG transfer) were transferred to duplicate selective plates. One plate (master) was stored at 4°C, and the other used for colony filter hybridization (26). The *pat* and *cry*IVB fragments were used as probes to detect RP4p, pSKTG or the chromosomal marker cassette KTG (28, 30). Both sequences were obtained from their cloning vectors by restriction with *Hin*dIII (*pat*) or *Xba*I (*cry*IVB). The 0.7 kb (*pat*) and 1.4 kb (*cry*IVB) fragments

were cut from agarose gels and purified by electro-elution. The colony blots were washed at highest stringency before exposure of X-ray film. Colonies reacting positively with the probes were further checked, to discriminate between indigenous transconjugants and surviving donor cells, by plating on King's B agar containing Rp. Since in previous work indigenous transconjugants which received RP4p or pSKTG have been identified to genus/species level (25, 28), this was not done here.

Statistics

Differences in colony forming unit counts between treatments were statistically analyzed using ANOVA at a level of significance of $P<0.05$, using the Genstat-5 programme. The overal LSD value for the experiments is given in the Figures.

RESULTS

Chromosome mobilisation on agar and in soil

In previous experiments RP4p had been transferred in filter matings with a frequency of 10^{-2} and mobilization of plasmid pSKTG by RP4p had occurred with similar frequencies (31). In the present filter mating experiments, RP4p was transferred from donor to homologous recipient with similar frequency (TABLE 2). RP4p present in *P. fluorescens* R2fmc could mobilize the chromosomally-inserted marker cassette with a low frequency ($4.5\ 10^{-8}$), comparable to literature (9). Parallel controls, in which donor and recipient were incubated separately, to assess the ocurrence of spontanous antibiotic resistance were negative. The presence of KTG in the transconjugants was confirmed by colony blotting and antibiotic resistance testing.

Previous work had shown that RP4p was transferred between introduced donor and recipient pseudomonads in sterile Ede loamy sand at a frequency around 10^{-4} (31).

Mobilization of the chromosomally-inserted marker cassette under the influence of RP4p present in the donor was studied in sterile Ede loamy sand and Flevo silt loam soils.

The introduced donor and recipient strains survived well (TABLE 3) in both soils. RP4p was transferred at a significantly higher frequency in Flevo silt loam soil than in Ede loamy sand soil. However, no mobilization of the KTG cassette was detected in either soil. The few colonies from Flevo silt loam appearing on the transconjugant-selective plates were, via extensive testing, shown not to represent genuine transconjugants, suggesting that the putative transfer of KTG was below detection.

Survival and transfer studies in non-sterile soils planted with wheat

P. fluorescens R2fR (RP4p) showed a slow but progressive decline in viabillity in Ede loamy sand over the time course of the experiment (FIG. 1A). In Flevo silt loam, up to day 17, cfu numbers did not decrease, and from day 17 until day 28 the cfu numbers decreased with 1 order of magnitude. Differences in survival rates in the two soils were very distinct; in Ede loamy sand (bulk soil), cfu counts dropped 4 orders of magnitude, while cfu counts in Flevo silt loam decreased only 1 order of magnitude during the 28 days. Cfu counts obtained from plates containing Rp were similar to those on plates containing Ap and Tc (difference< 0.1 Log unit), suggesting RP4p was stably maintained and the Tc and Ap resistance markers were expressed.

Transfer of RP4p to indigenous bacteria had been detected previously in the rhizosphere soil of wheat in short term experiments (25, 28). In this experiment, in Ede soil transconjugants were found on day 10 and 17, whereas on day 28 none were detected (TABLE 4). In Flevo silt loam, around 10^3 transconjugants per gram of soil were found throughout the experiment in rhizosphere and bulk soil.

Numbers of *P. fluorescens* R2fR (pSKTG) in Ede loamy sand (detected on plates with Gm) decreased in the first 10 days (FIG. 2A), after which cell numbers were relatively stable. The introduced strain showed a significantly enhanced survival in the rhizosphere as opposed to the bulk soil. Fluorescent cfu counts on plates containing only Km or Rp were slightly, but not significantly lower than counts on plates with Gm, suggesting that pSKTG was stably maintained and that the markers remained present and were expressed. Moreover, hybrization assays with the *cry*IVB probe of colonies appearing on Rp- or Km-containing agar plates confirmed the presence of the unselected marker in all colonies (50-100 per sampling time)

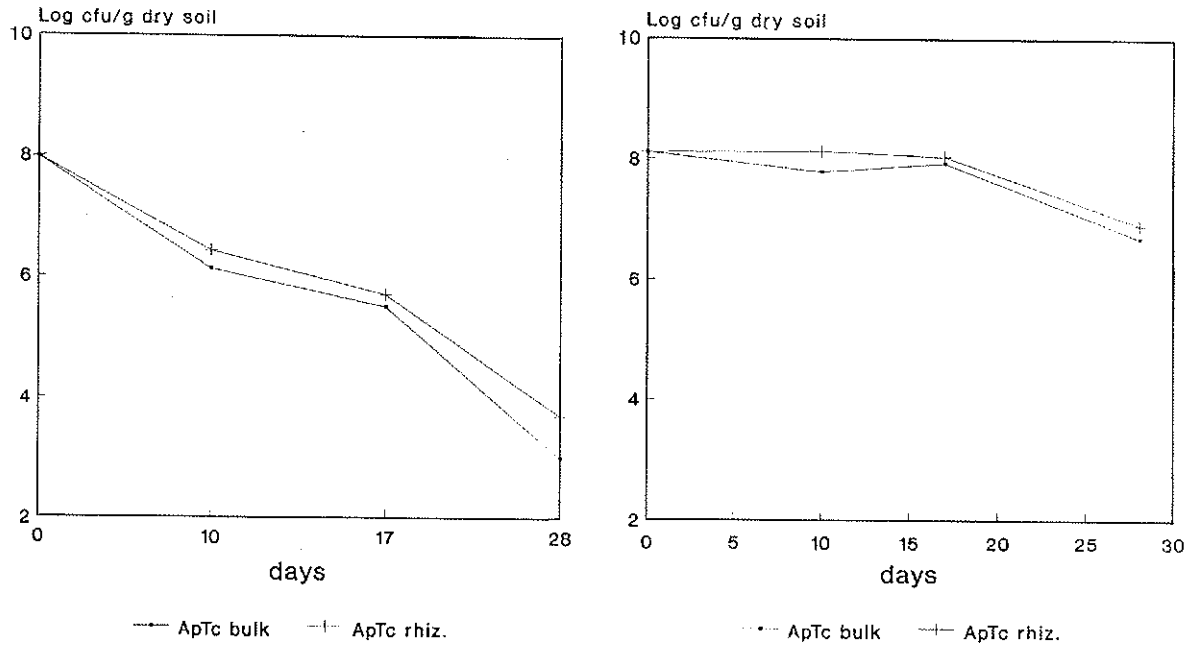


FIG. 1. Survival of *P. fluorescens* R2fR (RP4p) introduced into Ede loamy sand (A) and Flevo silt loam (B), planted with wheat. Cfu counts from plates containing Ap and Tc are given since these were identical to cfu counts on Rp-containing agar medium. LSD = 0.37.
ApTc bulk; ApTc rhizosphere.

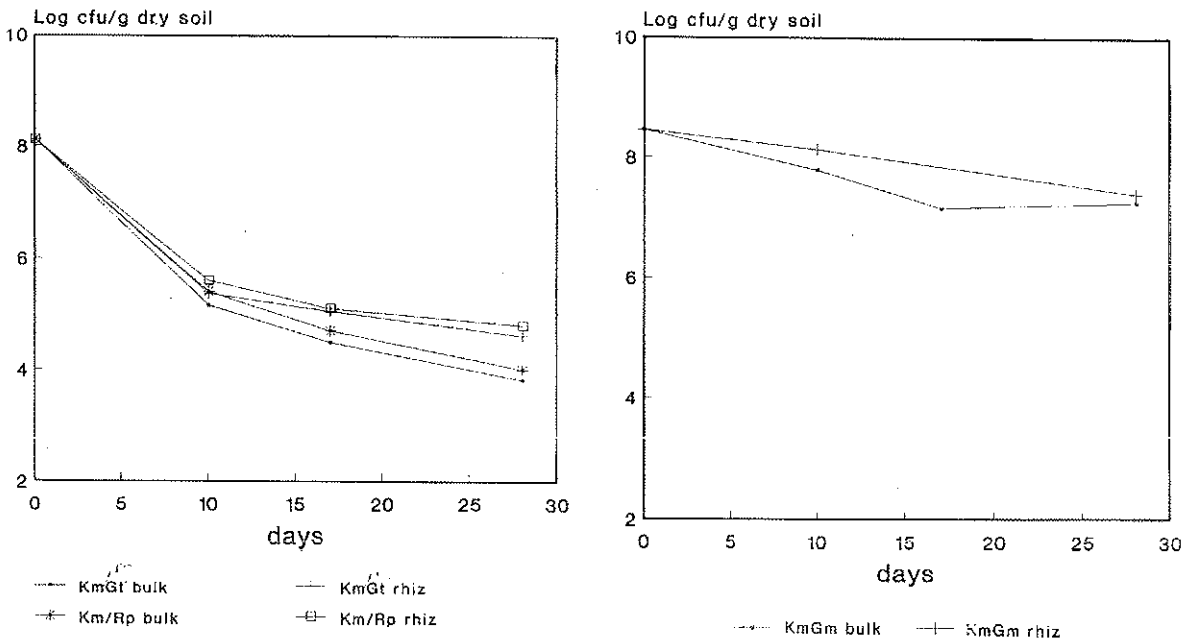


FIG. 2. Survival of *P. fluorescens* R2fR (pSKTG) in Ede loamy sand (A) and Flevo silt loam (B), planted with wheat. In Ede loamy sand, loss of expression of the Gm resistance gene is shown by the differences in cfu counts on media with and without Gm. In Flevo soil differences were within 0.1 Log unit. LSD = 0.37.
Rp/Km bulk; Rp/Km rhizosphere; Km/Gm bulk; Km/Gm rizosphere.

TABLE 4. Log number of indigenous transconjugants harbouring RP4p, pSKTG or the chromosomal KTG cassette, detected in a long-term soil experiment with three different *Pseudomonas fluorescens* strains with these elements as donors (for data on donor cfu see FIG. 1-3.)

Genetic element	Soil	Log cfu per g dry soil on day:							
		0		10		17		28	
		B	R	B	R	B	R	B	R
RP4p	ELS	[0]	3.1	[0]	3.3	[0]	[0]	[0]	
	FSL	2.3	2.6	3.3	3.4	ND	ND	3.7	3.6
pSKTG	ELS	[0]	[0]	[0]	[0]	[0]	[0]	[0]	[0]
	FSL	[0]	[0]	[0]	[0]	[0]	[0]	[0]	[0]
::KTG	ELS	[0]	[0]	[0]	[0]	[0]	[0]	[0]	[0]
	FSL	[0]	[0]	[0]	[0]	[0]	[0]	[0]	[0]

ELS: Ede loamy sand, FSL: Flevo silt loam, R: Rhizosphere, B: Bulk. [0]: below detection limit (approx. 10^2 cfu per g of dry soil).

tested. In Flevo silt loam, cfu numbers hardly declined, and loss of plasmid or expression was not detected (FIG. 2B). A rhizosphere effect on bacterial numbers was not apparent in this soil.

Indigenous bacteria containing the recombinant plasmid pSKTG were not detected in any of the 2 soils either in rhizosphere or bulk soil samples; the calculated limit of detection was slightly below 10^2 cfu per gram of soil.

Pseudomonas fluorescens R2fmc showed survival dynamics similar to that of *P. fluorescens* R2fR (pSKTG) in Ede loamy sand (Fig. 3A). Significant lower counts of fluorescent cfu were found on Gm-containing plates as compared to counts on Km- or Rp-containing plates, both in rhizosphere and in bulk soil samples, which could indicate loss of expression of the Gm resistance gene *aad*B. On day 28, this loss appeared to be significantly greater in bulk soil (87%) then in rhizosphere soil (25%), suggesting that the presence of plant roots diminished putative stress factors which affected the expression of *aad*B. There was only a slight decline in cfu counts in Flevo silt loam bulk and rhizosphere samples (Fig. 3B) and cfu counts from Km- plus Gm-containing plates did not differ significantly from those on Rp- or Km-containing plates, indicating that loss of expression of *aad*B did not occur in this soil.

Indigenous bacteria containing the marker gene cassette were not detected in either soil in rhizosphere and bulk soil samples, at a limit of detection of around 10^2 cfu per gram of soil.

Determination of expression level of aadB

To determine the level of loss of expression of the Gm resistance gene in Ede loamy sand, *P. fluorescens* R2fmc was plated 10 days after introduction (10^8 per gram of soil) onto King's B agar supplemented with an increasing amount of Gm (FIG. 4). Counts from plates containing Km without Gm were considered to represent 100% of the population. The percentage of the population able to grow in the presence of Gm was lower with increasing Gm concentrations, ranging from 70% at 10 mg/L to 30% at 50 mg/L. However, colonies first obtained on Km-supplemented plates without Gm were again able to grow in the presence of Gm (50 mg/L) upon subculturing. *P. fluorescens* R2fmc which had been starved for 10 days in 1/4 strength Ringers solution did not show any loss of *aad*B gene expression (FIG. 4), suggesting that starvation alone was not responsible for the phenomenon.

DISCUSSION

In filter matings, RP4p transferred with a frequency of 10^{-2} from a donor to a homologous recipient strain as found before (31). The frequency with which RP4p could mobilize the IncQ plasmid pSKTG appeared to be similar, at least in triparental matings on filters (31). This work revealed that the frequency with which RP4p could mobilize the chromosomally-inserted marker gene cassette was around 10^{-8} per donor. Mobilization of chromosomal DNA by conjugative plasmids has been described before (1, 9, 21), and gene transfer frequencies ranging from similar to ours to much higher, i.e. 10^{-3}, have been reported (9). However, the chromosome mobilization frequency of RP4 is always around 10^{-8}-10^{-9} (9). The rate-limiting step with RP4-mediated transfer of the chromosome probably is the concatenation (cointegration) of chromosomal and plasmid DNA. Thus, soil-borne plasmids of

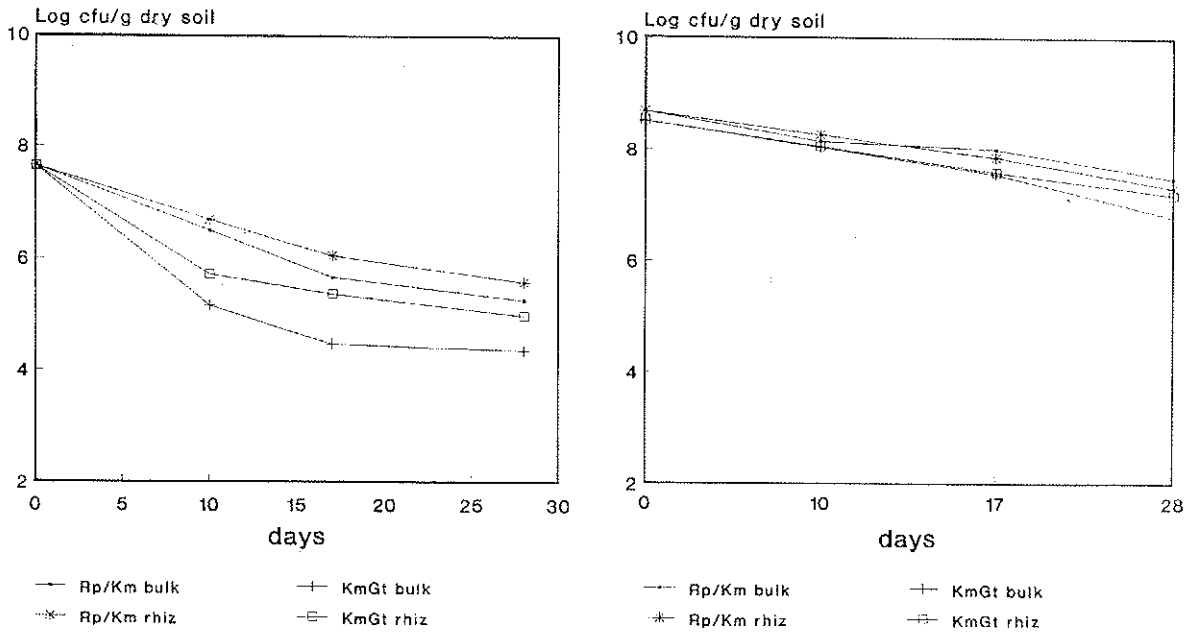


FIG. 3. Survival of *P. fluorescens* R2fmc introduced into Ede loamy sand (A) and Flevo silt loam (B), planted with wheat. Differences between cfu counts on plates with and without Gm in Flevo silt loam were below 0.1 Log unit. LSD = 0.37.
Rp/Km bulk; Rp/Km rhizospere; Km/Gm bulk; Km/Gm rhizosphere.

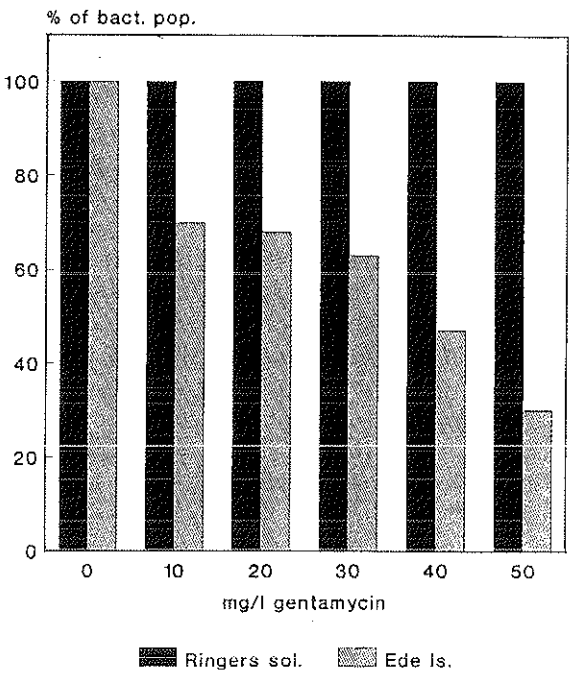


FIG. 4. Percentage of cfu counts of *P. fluorescens* R2fmc on King's B medium supplemented with increasing Gm concentrations, 10 days after introduction into Ede loamy sand or in 1/4 strength Ringers solution.

IncP or other mobilizing Inc groups might transfer chromosomally-inserted genes at frequencies in the range reported here (13, 19).

In sterile Ede loamy sand soil, RP4p had previously been shown to be transferred at a frequency of 10^{-4}, whereas mobilization of pSKTG by RP4p occurred at frequencies of 10^{-4} or 10^{-6}, depending on the initial localization of the mobilizer plasmid RP4p (31). However, soil type may drastically affect gene transfer frequencies (25). Here, we found that transfer of RP4p in sterile Flevo silt loam was 1000-fold higher than in sterile Ede loamy sand (TABLE 3), at similar donor and recipient cell numbers. Similarly, studies on the transfer of RP4p in non-sterile soil revealed highest numbers of indigenous transconjugants in Flevo silt loam (25). Soil parameters such as pH, clay minerals and organic matter content have been shown to influence conjugal gene transfer (24). Flevo silt loam might be a soil conducive to conjugal gene transfer due to its neutral pH and high clay content. However, environmental factors affecting conjugal transfer seem to interact in a complex way, both in soil (25) and artificial wastewater (14), often obscuring the elucidation of the effect

of each factor separately. In none of the 2 sterile soils, mobilization of the chromosomally-inserted marker gene cassette could be detected, not even in the presence of the mobilizer plasmid RP4p. As expected, the detection limit of roughly 50-100 cells per gram of soil in these experiments did not allow for chromosome mobilization in sterile soil to be detected. Transfer of chromosomal markers in sterile and non-sterile soils has been detected (14, 39) using an *Escherichia coli* Hfr donor strain and a co-introduced *E. coli* recipient strain. However, the rates of transfer reported by these authors are not indicative of frequencies which might be expected with non-Hfr strains. RP4-mediated chromosomal transfer in soil might take place at a maximal rate dictated by the rate-limiting step (frequency 4.5×10^{-8}) as argued above. Therefore, provided optimal cellular growth and contact possibilities in soil, chromosome mobilization might be expected to result in, at maximum, 1 transconjugant cell per gram of sterile (Flevo silt loam) soil with the donor and recipient population densities applied.

In both non-sterile soils planted with wheat, RP4p was transferred to indigenous bacteria, as found before (25, 28), indicating the rates at which cell-to-cell contact between the introduced and indigenous microorganisms was possible under given conditions in the soils. Conjugative plasmids occurring in bacteria in the environment (13; 19) might promote natural chromosome mobilization, probably at low frequencies. Assuming a frequency of occurrence in soil bacterial populations of such mobilizing plasmids of 10^{-6} per cell, a transfer frequency of 10^{-4} (indicating possibilities for successful cell-to-cell contacts; 28) and a chromosome mobilization frequency of 10^{-8} (TABLE 2), a total frequency of 10^{-18} per recombinant cell entering the soil might be expected.

The population dynamics of both *P. fluorescens* R2fR (RP4p) and *P. fluorescens* R2fR (pSKTG) in non-sterile Ede loamy sand exhibited a gradual decline in numbers and both plasmids seemed to be stably inherited, as found before for RP4p (Van Elsas and Trevors, 1990). *P. fluorescens* R2fmc revealed better survival in this soil as compared to the former strains (FIG. 3A). The metabolic load due to the extra genetic information was presumably smallest in the case of the chromosomal insert.

In Flevo silt loam soil, containing a high amount of clay minerals, all introduced strains survived markedly better then in Ede loamy sand, which confirms earlier work (12, 25, 33). The enhanced survival in clay soils as well as in Ede loamy sand amended with bentonite clay (12) has been attributed to an increase in numbers of small pores in which introduced bacteria are protected from predation (12). However, other factors, e.g. differences in pH, cannot be completely ruled out.

A significantly lower number of *P. fluorescens* R2fmc cfu's was obtained from Ede loamy sand on plates with Gm than on those without Gm. In all likelihood, temporarily reduced expression of the *aad*B gene caused this effect, since the gene was still present as shown by colony blots, and after growth on media without antibiotics, resistance was restored. Such apparent loss of expression of antibiotic resistance genes has been found before with several strains and antibiotic resistance genes in soil and aquatic environments (2, 3, 5, 8). Strains reisolated from the environment might be so weakened (injured) due to stressful environmental conditions that antibiotic resistance genes used as markers are temporarily not operational. Caldwell et al. (2) also showed that expression could be restored after resuscitation of the starved cells. We have no conclusive evidence that the lowered resistance against Gm is caused by loss of expression. For instance, Genthner et al. (5) found the lower numbers of cfu's on plates with certain antibiotics to be caused by a hypersensitive reaction of the bacteria after starvation. In our experiments, the loss of expression was prominent in *P. fluorescens* R2fmc, and it occurred to an insignificant extent in the pSKTG-containing strain. The higher copy number of the plasmid might have counteracted the loss of expression due to a gene dosage effect. Since loss of expression was not detected in cells from Flevo silt loam or in cells starved in Ringers solution, it was apparently caused by conditions specific for the Ede loamy sand soil other than starvation stress. These conditions might have caused a stress-induced response, invoking regulatory proteins in the introduced strain (38) which possibly affected the *aad*B promoter.

The results obtained here confirmed earlier suggestions that transfer of chromosomally inserted genetic elements (or even of those present on non-selftransmissible but mobilizable plasmids) does not detectably occur under soil conditions

conducive for the transfer of a selftransmissible element, RP4p. Therefore, when GEM release is cogitated, placement of the beneficial genes on the chromosome seems inherently safer than on mobilizable or selftransmissible plasmids. However, chromosomal inserts might be mobilized, at low frequencies, by selftransmissible plasmids, as shown here in the filter matings, and the experiments performed here with the non-sterile soils did not permit detection of these low-frequency events. It is conceivable that environmental pressures in soils selecting for specific (chromosomal) genes might favour bacteria harbouring these elements after transfer. Future work should focus on aspects of putative selection for the products of such low-frequency transfer events.

ACKNOWLEDGEMENTS

This work was sponsored by a grant from the Netherlands Integrated Programme for Soil Research, and partly by a grant from the CEC-STEP programme. We would like to thank Prof. Dr. J.A. van Veen and Prof. Dr. W.M de Vos for helpful discussions and Ir. S.B.G.J. Burgers for excellent help with statistics.

RESUMO

Estabilidade genética, transferência gênica e expressão de DNA heterólogo introduzido no genoma e em dois plasmídios de *Pseudomonas fluorescens* no solo

Neste trabalho estudamos a influência da localização de DNA heterólogo na estabilidade, expressão e transferência gênica, após a introdução de *Pseudomonas fluorescens*, R2f, no solo. Para tal, foram utilizadas três cepas contendo marcadores localizados, em elementos gênicos diferentes, isto é, um plasmídio auto-transmissível (RP4p), um plasmídio mobilizável (pSKTG) e um cassete gênico inserido no cromossomo (KTG). Experimentos "in vitro" de conjugação em filtro mostraram que o plasmídio auto-transmissível foi transferido com altas frequências (em torno de 10^{-2}) e que este plasmídio foi capaz de mobilizar pSKTG com frequências similares. Observou-se, também, a mobilização do cassete gênico, inserido no cromossomo, para uma cepa receptora, mas em baixas frequências (10^{-8}).

Não foi detectada, em solo estéril, a transferência do cromossomo, na presença de RP4p. Quando introduzidas em solos com diferentes texturas e plantados com trigo, as três cepas mostraram uma baixa sobrevivência em Flevo silt argiloso. Além disso, em Ede argiloso-arenoso, observou-se uma perda da expressão do gene *aadB*, que confere resistência a gentamicina e em Flevo silt argiloso isto não foi observado.

Em solos semeados com trigo foi detectado a transferência de RP4 para a população bacteriana indígena. Contudo, ao ser usada uma cepa doadora sem o plasmídio IncP não se detectou transconjuntantes contendo o plasmídio pSKTG e / ou cassete gênico inserido no cromossomo (KTG).

REFERENCES

1. Barth, P.T.- Plasmid RP4, with *Escherichia coli* DNA inserted *in vitro* mediates chromosomal transfer. *Plasmid* 2: 130-136, 1979.
2. Caldwell, B.A., Ye, C., Griffiths, R.P., Moyer, C.L. Morita, R.Y.- Plasmid expression and maintenance during long-term starvation-survival of bacteria in well water. *Appl. Environ. Microbiol.* 55: 1860-1864, 1989.
3. Devanas, M. A., Rafaeli-Eshkol, D., Stotzky, G.- Survival of plasmid-containing strains of *Escherichia coli* in soil: effect of plasmid size and nutrients on survival of hosts and maintenance of plasmids. *Curr. Microbiol.* 13: 269-277, 1986.
4. Fry, J.C., Day, M.J.- *Bacterial Genetics in the Natural Environment*. Chapman and Hall, London, 1982.
5. Genthner, F.J., Upadhyay, J., Campbell, R.P., Sharak Genthner, B.R.- Anomalies in the enumeration of starved bacteria on culture media containing nalidixic acid and tetracycline. *Microbiol. Ecol.* 20: 283-288, 1990.
6. Germida, J.J., Khachatourians, G.G.- Transduction of *Escherichia coli* in soil. *Can. J. Microbiol.* 34: 190-193, 1988.
7. Graham, J.B., Istock, C.A.- Genetic exchange in *Bacillus subtilis* in soil. *Mol. Gen. Genet.* 116: 287-298, 1978.
8. Griffiths, R.P., Moyer, C.L., Caldwell, B.A., Ye, C., Morita, R.Y.- Long-term starvation-induced loss of antibiotic resistance in bacteria. *Microbiol. Ecol.* 19: 251-257, 1990.
9. Haas, D., Reimmann, C.- Use of Inc P plasmids in chromosomal genetics. In: *Promiscuous plasmids of Gram-negative bacteria*. Thomas, C.M., ed.. Academic Press, London, 1989, pp. 185-206.
10. Henschke, R.B., Schmidt, F.R.J.- Plasmid mobilization from genetically enengineered bacteria to members of the indigenous soil microflora *in situ*. *Curr. Microbiol.* 20: 105-110.
11. Herrero, M., De Lorenzo, V., Timmis, K.N.- Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J. Bacteriol.* 172: 6557-6567, 1990.

12. Heijnen, C.E., Van Veen, J.A.- A determination of protective microhabitats for bacteria introduced into soil. *FEMS Microbiol. Ecol.* 85: 73-80, 1990.
13. Hill, K.E., Weightman, A.J., Fry, J.C.- Isolation and screening of plasmids from the epilithon which mobilize recombinant plasmid pD10. *Appl. Environ. Microbiol.* 58: 1292-1300, 1992.
14. Khalil, T.A., Gealt, M.A.- Temperature, pH, and cations affect the ability of *Escherichia coli* to mobilize plasmids in L broth and synthetic wastewater. *Can. J. Microbiol.* 33: 733-737, 1987.
15. Khanna, M., Stotzky, G.- Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNAase on the transforming ability of bound DNA. *Appl. Environ. Microbiol.* 58: 1930-1939, 1992.
16. Krasovsky, V.N., Stotzky, G. - Conjugation and genetic recombination in *Escherichia coli* in sterile and non-sterile soil. *Soil Biol. Biochem.* 19: 631-638, 1987.
17. Levin, M.A., Strauss. H.S.- *Risk assessment, in genetic engineering*. McGraw-Hill, New York, 1990.
18. Levy, S.B., Miller, R.- *Gene Transfer in the Environment*. McGraw-Hill, New York, 1989.
19. Lilley, A.K., Bailey, M.J., Day, M.J., Fry, J.C.- Natural transfer plasmids from sugarbeet rhizosphere. In: Abstracts of the Sixth International Symposium on Microbial Ecology, 6-11 Sept. 1992, Barcelona, 1992.
20. Lorenz, M.G., Wackernagel, W.- High frequency of natural genetic transformation of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorus source. *Appl. Environ Microbiol.* 57:1246-1251, 1991.
21. Pemberton, J.M., Bowen, A.R.St.G.- High frequency chromosome transfer in *Rhodopseudomonas sphaeroides* promoted by broad-host-range plasmid RP1 carrying mercury transposon Tn501. *J. Bacteriol.* 147: 110-117, 1991
22. Ramos-Gonzales, M-I., Duque, E., Ramos, J.L.- Conjugational transfer of recombinant DNA in cultures and in soils: host range of *Pseudomonas putida* TOL plasmids. *Appl. Environ. Microbiol.* 57: 3020-3027, 1991.
23. Reanney, D.C., Gowland, P.C., Slater, J.H.- Genetic interactions among microbial communities. *Symp. Soc. Gen. Microbiol.* 34: 379-421, 1983.
24. Richaume, A., Angle, J.S., Sadowski, M.J.- Influence of soil variables on in situ plasmid transfer from *Escherichia coli* to *Rhizobium fredii*. *Appl. Environ. Microbiol.* 55: 1730-1734, 1989.
25. Richaume, A., Smit, E., Faurie, G., Van Elsas, J.D.- Influence of soil type on the transfer of plasmid RP4p from *Pseudomonas fluorescens* to introduced recipient and to indigenous bacteria. *FEMS Microbiol. Ecol.* 101: 281- 292, 1992.
26. Sambrook, J., Fritsch, E.F., Maniatis, T.- *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y, 1989.
27. Sistrom, W.R.- Transfer of chromosomal genes mediated by plasmid R68.45 in *Rhodopseudomonas sphaeroides*. *J. Bacteriol.* 131: 526-532, 1977.
28. Smit, E., Van Elsas, J.D., Van Veen, J.A., De Vos, W.M.- Detection of plasmid transfer from *Pseudomonas fluorescens* to indigenous bacteria in soil by using bacteriophage FR2f for donor counterselection. *Appl. Environ. Microbiol.* 57: 3482-3488, 1991.
29. Smit, E., Van Elsas, J.D, Van Veen, J.A.- Risks associated with the application of genetically engineered microorganisms in terrestrial environments. *FEMS Microbiol. Rev.* 88: 263-278, 1992.
30. Smit, E., Van Elsas, J.D.- Conjugal gene transfer in the soil environment; new approaches and developments. In: *Gene transfers and environment*. Gauthier, M.J., ed. pp.79-93. Springer-Verlag, Berlin, 1992.
31. Smit, E., Venne, D., Van Elsas, J.D.- Mobilization of a recombinant IncQ plasmid between bacteria in soil via co-transfer or retro-transfer. *Appl. Environ. Microbiol*, 1993.
32. Top, E., Mergeay, M., Springael, D., Verstraete, W.- Gene escape model: transfer of heavy metal resistance genes from *Escherichia coli* to *Alcaligenes eutrophus* on agar plates and in soil samples. *Appl. Environ. Microbiol.* 56: 2471-2479, 1990.
33. Van Elsas, J.D., Dijkstra, A.F., Govaert, J.M., Van Veen, J.A.- Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. *FEMS Microbiol. Ecol.* 38: 151-160, 1986.
34. Van Elsas, J.D., Govaert, J.M., Van Veen, J.A.- Transfer of plasmid pFT30 between bacilli in soil as influenced by bacterial population dynamics and soil conditions. *Soil Biol. Biochem.* 19: 639-647, 1987.
35. Van Elsas, J.D., Trevors, J.T., Starodub, M.E.- Bacterial conjugation between pseudomonads in the rhizosphere of wheat. *FEMS Microbiol. Ecol.* 53: 299-306, 1988a.
36. Van Elsas, J.D., Trevors, J.T., Starodub, M.E.- Plasmid transfer in soil and rhizosphere. In: *Risk Assessment for Deliberate Releases*. Klingmüller, W., ed. pp. 89-99, Springer Verlag, Heidelberg, 1988b.
37. Van Elsas, J.D., Trevors, J.T.- Plasmid transfer to indigenous bacteria in soil and rhizosphere: problems and perspectives. In: *Bacterial Genetics in Natural Environments*. Fry, J.C. and Day, M.J., eds. Chapman and Hall, London, 1990, pp. 188-199..
38. Van Elsas, J.D., Van Overbeek, L.S.- Bacterial responses to soil stimuli. *In: Starvation in bacteria*. Kjellenberg, S., ed. Plenum Press, N.Y., 1993.
39. Weinberg, S.R., Stotzky, G.- Conjugation and recombination of *Escherichia coli* in soil. *Soil. Biol. Biochem.* 4: 171-180, 1972
40. Wellington, E.M.H. Van Elsas, J.D. eds. - Gene Transfer Between Microorganisms in the Natural Environment. Pergamon Press, London, 1992.
41. Wellington, E.M.H., Cresswell, N., Saunders, V.A.- Growth and survival of Streptomycete inoculants and extent of plasmid transfer in sterile and non-sterile soil. *Appl. Environ. Microbiol.* 56: 1413-1419, 1990.
42. Zeph, L.R., Onaga, M.A., Stotzky, G.- Transduction of *Escherichia coli* by bacteriophage P1 in soil. *Appl. Environ. Microbiol.* 54: 1731-1737, 1988.

Rev. Microbiol., São Paulo, **26**(3): 180-185, 1995

SALMONELLA SEROTYPES FROM SURFACE WATERS IN SAN LUIS, ARGENTINA

Ida Josefa Martinez de Cortínez[1]*
Lidia del Carmen Velasquez [2]
Maria Ester Escudero [2]
Maria Ines Caffer[3]
Mariana Fernandez Cobo[4]
Ana Maria Stefanini de Guzmán [2]

ABSTRACT

The presence of *Salmonella* was investigated in water samples taken once a month from rivers and reservoirs, over a one year period, using the Moore's submerged gauze technique. Pre-enrichment was performed in buffered peptone water for 24 h at 37 °C, and enrichment in tetrathionate-brilliant green and Rappaport broth for 24 h at 42 °C. Isolation was done by incubation for 48 h at 37 °C on brilliant green agar with 0.25% sodium desoxycholate and on bismuth sulfite agar. Suspected colonies were studied by classical biochemical and serological tests. *Salmonella* was recovered from 6.66% of the samples. *S. panama*, *S. newport*, *S. sandiego* and *Salmonella* IV, 40:Z_4,Z_{23}:- were detected, the latter serotype being isolated for the first time in Argentina. Isolates were obtained in the summertime. Microbial antibiotype was determined against seven antibiotics by the Kirby-Bauer method. Plasmids extracted as described by Birnboin and Doly were exposed to electrophoresis on agarose gel. The approximate molecular weights (MW) of the plasmids in megadaltons (MDa) were determined by comparison with known MW plasmids (*Escherichia coli* V 517). Plasmids of MW 40 MDa were detected in *S. newport* and plasmids of MW 17 MDa in *S. sandiego*. The 15 strains studied were susceptible to trimethoprim-sulfamethoxazole, gentamicin, tetracyclin, chloramphenicol, kanamycin and nalidixic acid. With respect to carbenicillin, six strains were resistant, seven strains showed intermediate susceptibility, and only two strains (those in which plasmids were detected) were susceptible to it. The systematic determination of epidemiological markers during outbreaks and in sporadic cases of salmonellosis are of interest in order to establish their geographical distribution. A microbiological surveillance of surface waters and the implementation of suitable wastewater purification procedures prior to their discharge into receiving waters is also a necessary measure.

Key words: *Salmonella* serotypes, waters, epidemiological markers.

[1] Cátedra de Microbiologia; [2]Cátedra de Bacteriologia I, Area de Microbiologia, Facultad de Química, Bioiquímica y Farmácia; Universidad Nacional de San Luis, Argentina; [3] Centro Nacional de Referência de *Salmonella*, *Shigella*, *Escherichia* y Vibrio; [4]División Bioquímica, Departamento de Virus. Instituto Nacional de Microbiologia "Dr. Carlos G. Malbrán"

* To whom correspondence should be sent, at the address: Area Microbiologia; Facultad de Química, Bioquímic y Farmácia. Universidad Nacional de San Luís. Chacabuco y Pedernera, 5700 San Luis, Argentina.

INTRODUCTION

The microbiological quality of surface waters used for human consumption, irrigation and recreation is an important public health issue, since these waters can become contaminated with pathogens. Salmonellosis manifests itself as a gastrointestinal syndrome caused by ingestion of food or water containing some *Salmonella* serotype. Serotypes which appear to be the most common ethiologic agents of human alimentary infections are, among others, *S. enteritidis, S. panama, S. typhimurium, S. oranienburg, S. stanley, S. heidelberg, S. branderburg, S. newport* and *S. derby*. The predominant role of these microorganisms as contaminants is supposed to be due to their persistence in normal and residual waters (9).

Surface water *Salmonella* has been widely investigated in Argentina, Serotypes such as *S. eteriditis, S. infantis, S. israel, S. mbandaka, S. panama, S. typhimurium* and *S.* sub species IV 18 Z_{36}:Z_{38}:- (4) have been isolated from the waters of Lujan River, which flows across a urban area of the Buenos Aires province (4). *S. zaiman*, a new world serotype, was isolated from Zaiman stream waters in Posadas, Misiones. *S. saphra, S. anatum, S. typhimurium, S. panama, S. agona, S. oranienburg, S. newport, S. derby, S. infantis, S. belem, S. heidelberg, S. bredeney* and others have been isolated from various streams in Posadas that receive sewage discharges (3).

The use of non-drinking water for irrigation is becoming common in areas where water is scarce. Such practice poses a serious threat to human health since it can become a source of food contamination with *Salmonella,* thus creating a never-ending infection cycle (4).

The aim of the present work was to determine the presence of *Salmonella sp* in two reservoirs and three rivers of the San Luis province, Argentina, all of which have important tourist facilities on their banks. In some cases wastewater is discharged into the river or reservoir waters either directly or after partial treatment. In other cases, sewage is disposed of in tanks, but a sandy soil allows the infiltration of microorganisms into the water courses. Plasmid profiles and susceptibility to antimicrobial agents were determined as epidemiological markers for the isolated *Salmonella* strains. These markers permit to interrelate factors such as transmission pathways, geographical distribution and common reservoir of origin.

MATERIALS AND METHODS

Samples

The river and reservoir samples were collected once a month over a year, always at the same site, in the San Luis province, Argentina. Sampling was performed according to Moore's submerged gauze technique (12) for one hour (1). Sixty samples were studied. Samples were kept under refrigeration until their analysis, which was performed within six hours of collection.

Procedure

Pre-enrichment was carried out by placing the submerged gauze in 100mL of buffered peptone water (Merck) (10). After incubation at 37°C for 24 hours, aliquots of 1mL were transferred to tubes containing 10mL of tetrathionate-brilliant green broth (Merck) and to bottles with 100mL of Rappaport broth, and further incubated at 42 + 0.5°C for 24 hours. From these cultures, isolation was performed by incubation for 48 h at 35°C on brilliant green agar (Merck) with 0.25% sodium desoxycholate (Sigma) (BGA-D) (1) and on bismuth sulfite agar (Oxoid) (BSA). Plates were examined daily, and colonies suspected to be *Salmonellae* were inoculated onto iron triple sugar agar. Cultures on this agar medium with a typical *Salmonella sp* reaction were screened in urea and fenylalanine media. Urea and fenylalanine-negative cultures were analyzed by classical biochemical tests. They were then serologically confirmed with polyvalent antisera provided by the Serum and Antigen Division of the National Institute of Microbiology "Dr. Carlos G. Malbrán". Strains exhibiting biochemical characteristics and serology typical of *Salmonella sp.* were sent to the National *Salmonella*, *Shigella*, *Escherichia* and *Vibrio* Reference Center of the National Institute of Microbiology "Dr. Carlos G. Malbrán", Argentina, and to the Pasteur Institute, Paris, France, for determination of their antigenic structure.

Determination of epidemiological markers

Plasmid profile and susceptibility to antimicrobial agents were established as epidemiological markers for all the isolated strains. Plasmids were extracted by the Birnboin-Doly's method (5) and were run on a 0.8% horizontal agarose gel in tris-borate-EDTA buffer (TBE), at 100 v. for 4 hours. Gels were stained with ethidium bromide and transilluminated under UV.Their MW was determined and expressed in megadaltons (MDa). *Escherichia coli* V 517, which contains plasmids of 35; 5.2; 3.5; 3; 2.25; 1.7; 1.5 and 1.25 MDa, was used as MW control. Antibiotypes were determined according to Kirby-Bauer's method (2) against seven antimicrobial agents: trimethoprim-sulfamethoxazole, kanamycin, nalidixic acid, and carbenicillin. The chi-square test was used for the statistical analysis of results (14).

RESULTS

Salmonella sp was identified in 4 (6.66%) out of a total of 60 samples analyzed . *S. panama* was found in the waters of one reservoir and one river, and *S. newport*, *S. sandiego* and *Salmonella* sub-species IV, 40:Z_4,Z_{23}:- were detected in river waters (TABLE 1). These serotypes were obtained only after enrichment in Rappaport broth and isolation on both BGA-D and BSA (TABLE 2). All the identifications were accomplished during the summer months, between November 1992 and February 1993. Plasmids of MW 40 MDa were detected in *S. newport* and of MW 17 MDa in *S. sandiego* (FIGURE 1). All the strains were susceptible to trimethoprim-sulfamethoxazol, gentamicin, tetracycline, chloramphenicol, kanamycin and nalidixic acid. As regards carbenicillin, six strains exhibited resistance, seven presented intermediate susceptibility and only two strains, those in which plasmids had been detected, were classified as susceptible.

TABLE 1. Identification of isolates

Origin	Number of isolates	Serotypes
Grande River	4	*Salmonella* sub-species IV, 40:Z_4,Z_{23}:-
Trapiche River	3	*Salmonella panama*
Volcan River	2	*Salmonella newport* and *S. sandiego*
La Florida reservoir	-	
Cruz de Piedra reservoir	6	*Salmonella panama*

TABLE 2. Number os *Salmonella sp* isolated with respect to culture media used

Pre-enrichment medium	Enrichment media	Isolation media	
		Brilliant green agar with sodium desoxycholate	Bismuth sulfite agar
Buffered peptone water	Brilliant green-tetrathionate broth	-	-
	Rappaport broth	2^{CP} 3^{T} 2^{RG} 1^{V}	4^{C} 2^{RG} 1^{V}

CP = Cruz de Piedra reservoir; T = Trapiche river; V = Volcan river; RG = Grande river

DISCUSSION

It has been noted that the efficiency of *Salmonellae* isolation from surface waters is enhanced by pre-enrichment in buffered peptone water (1,10). In the present study, unmodified Rappaport broth was used (1), which proved to be more efficient than Muller and Kauffman brilliant green tetrathionate broth, the latter probably requiring a longer incubation time (approximately 72 hours) to increase its efficiency (4). Incubation in these enrichment broths was carried out at 42+ 0.5°C since several *Salmonella* serotypes do not grow at 43.5°C (16).

Both BGA-D and BSA proved to be equally efficient as isolation media for the recovery of *Salmonella sp* ($p=0.05$), a finding which is in agreement with results reported by other authors (7). Serotype *S.* sub-species IV, 40:Z_4,Z_{23}:- was obtained from 6.66% of the *Salmonella sp.* isolates, being this its first isolation in Argentina.

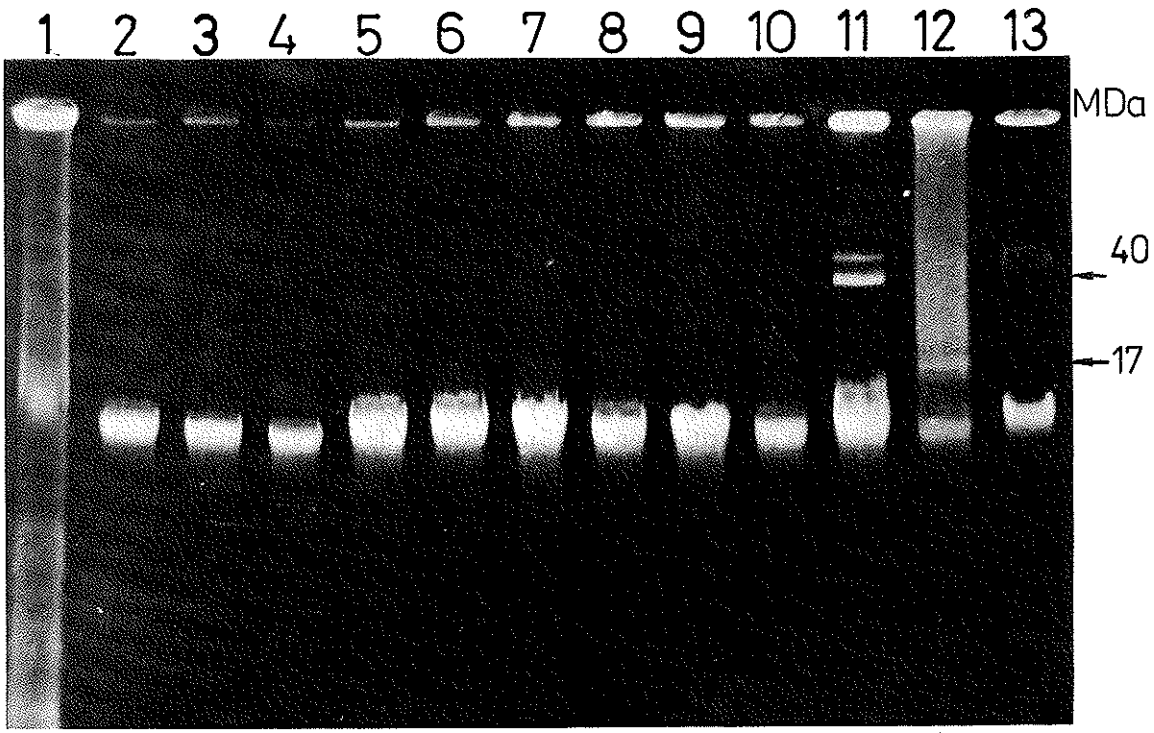


FIGURE 1. 0.8% Horizontal agarose gel. Lines 1 and 13: plasmids from the *E. coli* V517 control strain; lines 2 to 10: *Salmonella sp* strains which did not exhibit plasmids; line 11: 40 MDa plasmid detected in *S. newport*; line 12: 17 MDa plasmid detected in *S. sandiego*. Sizes are given on the right in megadaltons.

Other authors (11.15) have found that plasmid profile (PP) and phage types (PT) are successfully employed as epidemiological indicators for *S. enteriditis* subtyping. Susceptibility to antimicrobial agents, on the other hand, has not been as efficient as PP and PT for typing *S. enteriditis*, as outbreak and sporadic isolates were non-resistant (13).

Rodriguez *et al* (13) have reported PP and PT to be the most efficient epidemiological indicators. In the present study, PP and patterns of bacterial resistance to antibiotics were not comparable, since most of the strains were susceptible and the two strains carrying plasmids were susceptible to all the antibiotics. The plasmids of these two strains may not be related to resistance to antimicrobial agents.

In studies on *Salmonella enteriditis* outbreaks in San Luis, Argentina (6,8) and on sporadic cases in which *S. newport* and *S. sandiego* were isolated from human feces (personal communication from Dr. Bonardello from the Public Health Laboratory of San Luis), epidemiological markers such as PP and resistance to antimicrobial agents were not determined. These, however, could prove to be useful for the epidemiological follow-up of human and animal-associated infections and infections transmitted by water and regional foods.

In the present work, the study area comprised La Florida and Cruz de Piedra reservoirs and their respective tributaries: Grande and El Trapiche rivers for the former reservoir and Volcan river for the latter. The isolation could only be performed during the summer, when higher temperatures and inflow of tourists are probable intervening factors. Highly significant differences ($p<0.001$) were observed between the frequencies of serotypes isolated during the summer and those found during the rest of the year. The comparison of frequencies was done using the chi-square statistical method (14).

During the period between November 1992 and February 1993, values for fecal contamination

in the studied reservoires and rivers, as determined by the NMP of fecal coliforms (FC) in 100mL, ranged from 60 to 90 for La Florida reservoir and from 140 to 170 for its tributaries. At the Cruz de Piedra reservoir, values varied between 130 and 170 whereas those of its tributaries ranged from 240 to 300.

During the months of March and April 1992, the heavy rain probably had a diluent effect. The results obtained show the presence of various serotypes of *Salmonella* sp. in San Luis rivers and reservoirs, and constitute a warning for the need to perform microbiological monitoring and to implement appropriate methods of purification of wastewater prior to their discharged into water courses.

ACKNOWLEDGMENTS

We thank Prof. Isabel Giménez for her collaboration with the statistical analysis, and Nilda Tascón and Alfredo Villegas for their technical assistance.

RESUMO

Serovariedades de *Salmonella* em águas superficiais em San Luís, Argentina.

Foi investigada a presença de *Salmonella* em amostras de água colhidas pela técnica de Moore, em rios e açudes, durante um ano. Realizou-se um pré-enriquecimento em água peptonada tamponada e um enriquecimento em caldo tetrationato-verde brilhante e caldo Rappaport. Isolou-se em ágar verde brilhante com desoxicolato de sódio a 0.25% e em ágar sulfito de bismuto. As colônias suspeitas foram estudadas mediante provas bioquímicas clássicas e sorológicas. Recuperou-se *Salmonella* em 6.66% das amostras. Isolou-se *S. panama*, *S. newport*, *S. sandiego* e *Salmonella* sub-espécie IV, 40:Z_4, Z_{23}:-, esta última serovariedade constitui o primeiro isolamento na Argentina. Determinou-se o antibiograma pelo método de Kirby-Bauer. As 15 cepas isoladas foram sensíveis a trimetroprima-sulfametoxazol, gentamicina, tetraciclina, cloranfenicol, kanamicina e ácido nalidíxico; quanto à carbenicilina, 6 cepas foram resistentes, 7 mostraram sensibilidade intermediária e só 2 foram sensíveis. Foram determinados perfis plasmídicos pela técnica de Birnboin e Doly. Os pesos moleculares (PM) dos plasmídeos em megadaltons (MDa) foram determinados por comparação com plasmídeos de PM conhecidos. Foram detectados plasmídeos de PM 40 MDa em *S. newport* e de PM 17 MDa em *S. sandiego*. Seria de interesse a determinação sistemática de marcadores epidemiológicos em surtos e casos esporádicos de salmonelose com o fim de estabelecer sua distribuição geográfica na zona estudada da província de San Luis, assim como também a necessidade de uma vigilância microbiológica das águas superficiais e a implementação de tratamentos de depuração de águas cloacais adequados antes de serem despejadas nos cursos de água.

REFERENCES

1. Anselmo, R.J., Barrios, H., Viora, S., Lorent, B., Eiguer, T., Caffer, M.I., Fliess, E. Estudio comparativo de cuatro métodos de aislamiento de salmonelas de aguas superficiales. *Rev. Arg. Microbiol.* 21: 127-132, 1989.
2. Bauer. A., Kirby, W.M.M., Sherris,J.C.; Truck, M. Antibiotic susceptibility testing by a standardized single disk method. *Am J. Clin Pathol.* 45: 493-496, 1966.
3. Benassi, F.O., Martinez Vazquez, F.; Eiguer, T. *Salmonella:* su incidencia en aguas del aroyo Zaimán. *Rev. Arg. Microbiol.* 15: 169-175, 1983.
4. Benassi, F.O. Martinez Velazquez, F., Eiguer, T., Berdersky, S.; Martos, M.A. Aislamiento de nuevas serovariedades de *Salmonella* en cursos de aguas. *Rev. Arg. Microbiol.* 17: 149-155, 1985.
5. Birnboin, H.C.; Doly, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* 7: 1513-1523, 1979.
6. Bonardello, N. *et al.* Brotes de salmonelosis en la ciudad de San Luis. *Bol. Asoc. Arg. Microbiol.* Nº 80, 1989.
7. Cortinez, I.J.M., Velazquez, L. del C., Eiguer, T., Caffer, M.I.; Guzmán, A.M.S. Determinación de la calidad higiénico-sanitaria de fideos frescos. *Rev. Arg. Microbiol.* 20: 19-200, 1988.
8. Eiguer, T., Caffer, M.I.; Fronchkowsky, G.B. Importancia de la *Salmonella enteritidis* en brotes de enfermedades transmitidas por alimentos en Argentina, años 1986-1988. *Rev. Arg. Microbiol.* 22: 41-46, 1990.
9. Fernandez, N.C.; Frade, A.H. Salmonelosis. *Infect. & Microbiol. Clin.* 3: 79-80, 1991.
10. Fricker, C.R. A comparison of isolation procedures for *Salmonella* from polluted water using two forms of Rappaport's medium. *J. Appl. Bacteriol.* 56: 305-309, 1984.
11. Mayer, L.W. Use of plasmid profiles in epidemiologic surveillance of disease outbreaks and in tracing the transmission of antibiotic resistance. *Clin. Microbiol. Rev.* 1: 228-243, 1988.
12. Moore, B. The detection of paratyphoid carriers in towns by means of sewage examination. *Monthly Bull. Minist. Hlth.*

(Lond). 7: 241-248, 1948.

13. Rodriguez, D.C., Cameron, D.N., Puhr, N.D., Brenner, F.W., St. Louis, M.E., Wachsmuth, I.K.; Tauxe, R.V. Comparison of plasmid profiles, phage types and antimicrobial resistance patterns of *Salmonella enteritidis* isolates in the United States. *J. Clin. Microbiol.* 30: 854-857, 1992.
14. Snedecor, G.W.; Cochran, W.G. Métodos Estatísticos. 10ma Edición. Ed. Compañia Editorial Continental. México. Cap. I, P. 39-48, 1984.
15. Taylor, D.N. Wachsmuth, I.K., Yung-Hui, S., *et al.* Salmonellosis associated with marijuana, a multistate outbreak traced by plasmid fingerprinting. *N. Engl. J. Med.* 306: 149-1253, 1982.
16. Van Schothorst, M. Standartization of microbiological methods. The *Salmonella* example. *The Veterinary Quarterly*. 9: 332-338, 1987.

Rev. Microbiol., São Paulo, **26**(3): 186-191, 1995

OCCURRENCE OF *LEGIONELLA SP* IN WATER SAMPLES FROM MAN-MADE SYSTEMS OF SÃO PAULO - BRAZIL.

Vivian Helena Pellizari[1]
Maria Therezinha Martins[1]

ABSTRACT

Information on the presence of *Legionella sp* in the Brazilian environment is rather scarce. The aim of this study was to access the occurrence of *Legionella* sp in water from houses, public buildings and nosocomial and industrial systems of the city of São Paulo, Brazil. Sixty nine water samples from cooling towers, evaporative condensers, hot water supplies, showers, tap waters, reservoirs, hospital humidifiers and other sampling sites from public buildings, houses, hospitals and industries were analyzed. Samples were submitted to thermic and acid treatment in parallel and were next inoculated onto BCYE-Agar with or without antibiotics. Untreated samples were also studied. Suspected colonies of *Legionella sp* were reisolated and analyzed by direct immunofluorescence, and then inoculated into guinea pigs and embryonated eggs. Six (8.7%) of the sixty nine samples analyzed were positive for *Legionella sp*. A total of nineteen colonies were obtained, being eleven of *L. pneumophila* serogroup 1, seven of *L. pneumophila* serogroup 6 and one of *L. bozemanii*. In conclusion, the data demonstrate the presence of *Legionella sp* in São Paulo's environment, which constitutes a risk to public health and points to the need for a reliable, simple and economic method of water surveillance.

Key words - *Legionella pneumophila, Legionella* sp, water.

INTRODUCTION

The discovery of bacteria of the genus *Legionella* took place during the investigation of an explosive outbreak of pneumonia among delegates of the annual convention of the American Legion, Pennsylvania Division, in July 1976 (McDade *et al*, 1977). It is now well recognized that Legionnaires' disease occurs worldwide and that members of the family *Legionellaceae* are ubiquitous in the water environment. The two more common forms of legionellosis are Pontiac Fever and Legionnaires' disease, water being the main source of *Legionella* infection (Fraser and McDade, 1979; Brenner *et al*, 1984).

The first association between *Legionella* and water distribution systems occurred when the organism was isolated from shower fixtures of the rooms of two renal transplant patients. Following that episode, outbreaks of waterborne legionellosis have been described. The sources mentioned are showers, faucets, room humidifiers, reservoirs, hot water tanks, air conditioning, cooling towers and other man-made systems (Steele *et al*,1990; Barbaree, 1987; Woo *et al*, 1986; Stout *et al*, 1985).

[1] Laboratório de Microbiologia Ambiental, Instituto de Ciências Biomédicas, Universidade de São Paulo, Brasil.
Address: Instituto de Ciências Biomédicas II. Universidade de São Paulo. Av. Prof. Lineu Prestes 1374. São Paulo. S.P. Brasil. CEP 05508-900, Fax: 005511-813 0845. E. MAIL-VHPELLiZ@icb2.biomed.USP.BR

In Brazil, Pereira Gomes (1989) and Mazzieri (1990) described the isolation of *Legionella pneumophila* serogroup 1 from clinical samples. Levin *et al* (1991) published the isolation of *Legionella pneumophila* serogroup 1 from air conditioning water and *L.anisa* from showers in a hospital. These data were associated with an outbreak of pneumonia in the Renal Transplant Unit of the same hospital.

The aim of this study was to access the distribution of *Legionella* sp in water samples from houses, public buildings and nosocomial and industrial systems of the city of São Paulo, Brazil.

MATERIALS AND METHODS

Samples: A total of sixty nine water samples (1L) were analyzed according to the "Standard methods for the examination of water and wastewater", 17ed (Anon. 1989) and Dutka *et al* (1984). Samples were collected from man-made systems at different places in the city of São Paulo, Brazil. A total of thirty five water samples were collected from eight different hospitals at the following sampling points: reservoirs, hot water tanks, nebulizers, faucets (hot and cold waters), showers (central heating and electrical system), cooling towers, drinking fountains and one vacuum system. Seven samples from cooling towers, five water samples from the condensing water inter outlet of the air conditioning and two samples from cooling towers were collected at two different industries. Twenty samples from drinking fountains, cooling towers and water tanks were collected at three office buildings. Residential samples were collected from seven different showers (electrical system or central heating).

Sample concentration and bacteriological tests: Water samples collected for *Legionella* isolation were filtered (1L) through 0.8μm, 0.6μm and 0.45μm polycarbonate membrane filters (Nucleopore). After concentration, the membranes were placed face down in 10mL of the filtered water samples and sonicated for 10 min. Next, portions of the sonicated samples were submitted to acid (incubation for 10 min with a pH 2 solution containing 0.2mL HCl/KCl and neutralization to pH 6.9) and heat (incubation at 50°C for 30min) treatments. Then, 0.5 mL aliquots of treated samples were plated in triplicate by the spread-plate technique onto BCYE agar (Buffered Charcoal Yeast Extract Agar (Difco) amended with L-cysteine and Ferric Pyrophosphate) and BCYE agar containing cephalothin, colistin, vancomycin, cycloheximide (BCYE-CCVC) or glycine, vancomycin-Polymixin B (BCYE-GVP) at the concentrations described by Feeley *et al* (1979). Samples not submitted to filtration or heat and acid treatment were inoculated directly onto the BCYE, BCYE-CCVC and BCYE-GVP agar media (Dutka and Ewan, 1983 ; Dutka *et al*, 1984).

As a control, a strain of *Legionella pneumophila* serogroup 1 was inoculated in one aliquot of the water samples (10^6 CFU/mL) and cultured on the same agar media described above. The plates were incubated for 10 days at 37°C. All the typical *Legionella* colonies (ground-glass appearance) were transferred to BCYE agar with and without L-cysteine and ferric pyrophosphate, subjected to Gram stain and tested for catalase, oxidase, gelatinase, urease and glucose utilization (Brenner *et al*, 1984). Colonies that showed positive results for *Legionella* sp were considered "suspected *Legionella* colonies", and were finally evaluated by Immunofluorescence and inoculation into guinea-pigs and embrionated eggs for confirmation.

Immunofluorescence test: Suspected colonies were confirmed by Direct Immunofluorescence Antibody stain (DFA) against specific antisera (Central Public Health Laboratory - Colindale) for nine species and eight different serogroups of *Legionella*: *L. pneumophila* serogroup 1,2,3,4,5,6,7 and 8, *L.dumoffi, L.longbeache, L.jordanis, L. wadsworthii, L.bozemanii, L.oakridgensis, L.micdadei* and *L.gormanii* (Wilkinson *et al*, 1979).

Inoculation of guinea pigs and embryonated eggs: Inoculation was performed according to McDade (1979). Concentrations of cells from the suspected colonies were adjusted to 10^6 UFC/mL and 1mL of each sample was injected intraperitoneally into two adult male guinea pigs (mean weight = 600 g), The animals were observed daily for symptoms of disease. After sacrifice, a portion of each guinea pig's spleen was removed, ground and suspended at 10% in PBS. These suspensions were diluted to 1% and inoculated into 7-day-old embryonated eggs. After incubation, the yolk sac membrane was separated. One portion was submitted to Gimenez Stain and another portion was diluted to 5% and tested by DFA.

RESULTS

Six samples collected from two hospitals (TABLE 1) and one from an office building (TABLE 2) revealed nineteen *Legionella* sp isolates when submitted to DFA. As shown in TABLES 1 and 2, the prevalence of *L. pneumophila* serogroup 1 was observed, with eleven isolates, followed by *L. pneumophila* serogroup 6, with seven isolates. One isolate positive for *L. bozemanii* was also detected.

TABLE 1. Confirmation of the presence of *Legionella sp* by DFA in water samples from two different hospitals.

Sampling points	Hospital	Number of isolates	DFA
Vacuum System	A	1	*L.pneumophila* sg 6
Nebulizer-TPU	B	1	**L.pneumophila* sg 1
Electric showers nursery	B	3	*L.pneumophila* sg 6

* Positive by Gimenez Stain
TPU = Terminal Patient Unit

TABLE 2. Confirmation of the presence of *Legionella sp* by DFA in water samples from one office building.

Sampling points	Number of isolates	DFA
Cooloing Tower 1	3	*L.pneumophila* sg 1
Cooling Tower 2	2	*L.pneumophila* sg 1 *1,2
	3	*L.pneumophila* sg 6
Cooling Tower 3	5	*L.pneumophila* sg 1 *1,2
	1	*L.bozemanii*

*1 Two positive samples by Gimenez stain.
*2 One positive sample by DFA after inoculation in guinea pigs.

When the nineteen isolates were inoculated into guinea pigs and embrionated eggs for confirmation, only two of them showed positive results by DAF, both being specific for *L. pneumophila* serogroup 1. The Gimenez stain and DAF gave positive results for four samples on analysis of yolk sac membranes.

Legionella sp was not detected in domestic or industrial water samples. TABLES 3 and 4 display the numbers and percentages of positive *Legionella sp* isolations in relation to the methodology of concentration, treatment and culture media utilized. It should be pointed out that both untreated water and culture media without antibiotics provided a better recovery of *Legionella sp.*

TABLE 3. Percentage and numbers of positive *Legionella sp* isolations in relation to the methodology of concentration and treatment utilized.

Procedure	Methodology	Number of *Legionella* isolates	%
1- Concentration of water sample:			
	Membrane Filters	10	52.6
	without concentration	09	47.4
n= 19			
2-Treatment of water sample:			
	acid	02	10.5
	termical	02	10.5
	without treatment	15	79.0
n= 19			

TABLE 4. Percentage and numbers of positive *Legionella sp* isolations in relation to the culture media utilized.

Culture Media	Number of *Legionella* Isolates	Percentage
BCYE-GVP AGAR	05	26.3
BCYE-CCVC AGAR	06	31.6
BCYE AGAR	08	42.1

BCYE - Buffered Charcoal Yeast Extract Agar.
GVP - Glycine, Vancomycin, Polymixin B.
CCVC - Cephalotin, Colistin, Vancomycin, Cyclohexamide.

DISCUSSION

This study employed the methodologies described by several authors to detect *Legionella sp* in water samples from man-made systems, including the utilization of acid and heat treatment to eliminate background growth (Dutka and Ewan, 1983; Dutka and Ewan, 1986; Tobin *et al*, 1986; Roberts *et al*, 1987).

The results showed that only 8.7% (6 samples) of the water samples analyzed were positive for the presence of *Legionella sp.* This low frequency of *Legionella* sp occurrence corroborates the results

presented by Joly *et al* (1983); Dutka *et al* (1984) and Dutka and Ewan (1986).

A total of nineteen isolates were obtained from two hospitals and one office building. *Legionella pneumophila* serogroups 1 and 6 and *L. bozemannii* were isolated from the cooling towers of an office building (TABLE 2). Hospital A (TABLE 1) presented 1 *L.peumophilla* serogroup 6 isolate in the vacuum system. In Hospital B, *L.pneumophila* serogroups 6 and 1 were detected in the electric shower and nebulizers, respectively. These positive samples originate from the water distribution systems of São Paulo, which receive complete treatment and final chlorination. The cooling towers sampled were being treated with biocides. The municipal water system, cooling water and reservoirs of warm water can act as efficient bacterial amplifiers, allowing small numbers of bacteria to multiply and colonize the systems. When present in biofilms, these bacteria can be more resistant to biocides and favor the growth of *Legionella sp* (Barbaree *et al*, 1986; Wadowsky *et al*, 1991). Water from the vacuum system of Hospital B was stagnant due to repairs in the building. This condition can also facilitate *Legionella* growth (Voss *et al*, 1985).

Another explanation for the low frequency of *Legionella sp* in our waters relates to the material used for the construction of water distribution networks of the city of São Paulo, which are mainly made of concrete and PVC but not of Iron, an important growth factor for the microorganism (Brenner *et al*, 1984; Habitch and Muller, 1988). Perhaps the high number of *Legionella sp* reported in Europe and other countries could be related to the use of iron pipelines. The presence of *Legionella pneumophila* in the water samples described in this study were not associated with cases of Legionnaire's disease. These data corroborate the results reported by Bartlett *et al* (1983).

Considering the methodology of isolation, it can be observed (TABLE 3) that there was a prevalence of positive results when samples were analyzed without previous treatment (89.5%) and in culture media without antibiotics (42.1%). Some important factors that interfere with *Legionella sp* isolation have been reported by other authors. For example, the stress imposed on the microorganism by membrane filtration, sonication and acid treatment, the possible presence of "viable but non culturable" *Legionella sp* in our environment (Hussong *et al*, 1987), and the deleterious action of antibiotic on the stressed bacteria.

The Direct Fluorescence Antibody (DFA) technique has been used by several authors (Cherry *et al*, 1979; McKiney *et al*, 1979; Joly *et al*, 1986; Wilkinson *et al*, 1990). DFA showed to be most sensitive for the detection of *Legionella sp* in environmental samples, but some false positive results have been described (Edelstein, 1980; Orrison *et al*, 1983).

In this study, DFA was not used to evaluate water samples directly but to confirm suspected isolates. The prevalence of serogroup 1 was observed, and only one strain of *L. bozemanii* was isolated. This specie was previously detected from water samples by Ortiz-Roque and Hazen (1987) and Lee and West (1991). The utilization of guinea pigs and embrionated eggs for confirmation of suspected colonies was not successful. We observed that, of the nineteen strains inoculated, only four were recovered after verification by Gimenez stain and DFA in yolk sac membrane and guinea pig spleens. Fliermans *et al*(1981) showed 100% positivity for samples analyzed by DFA and only 4% positivity by inoculation into guinea pigs. Therefore, our data shows that confirmation by inoculation of guinea pigs and embrionated eggs is not a satisfactory procedure and can be substituted for growth in artificial media and DAF.

In conclusion, this study demonstrates the presence of *Legionella sp* in the environment of São Paulo, Brazil, which may constitute a public health hazard, putting at risk particularly the population of susceptible individuals. One outbreak of *Legionellosis* was reported in São Paulo. Studies in our laboratory (unpublished data) also showed the presence of *Legionella pneumophila* in the hot water tank of a hospital building when the outbreak occurred. The findings also point to the need for a reliable, simple and economic method for the control of *Legionella sp* in water samples. The recently described molecular biology techniques for the detection of *Legionella* isolates could be a more sensitive, specific and economical means of surveillance *Legionella sp* in the environment.

ACKNOWLEDGMENTS

We would like to thank Maria Izabel P. Serrano, Margareth Del Bianchi and Debora M.M.

Pedroso for technical assistance and Dr. Jose A. Jerez for his help with the work with embryonated eggs . This research was supported by FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo). The author for correspondence was also awarded with a research grant by the General Society for Microbiology.

RESUMO

Ocorrência de *Legionella* spp em águas provenientes de residencias, prédios públicos e de ambientes hospitalares e industriais de São Paulo - Brasil.

O objetivo deste trabalho foi pesquisar a ocorrência de *Legionella* sp em águas residenciais, de prédios públicos e de ambientes hospitalares e industriais. Foram analisadas sessenta e nove amostras de água procedentes de torres de refrigeração, caldeiras, chuveiros, torneiras, reservatórios e outros pontos específicos de prédios públicos, residências, hospitais e indústrias. As amostras foram submetidas à concentração e em seguida aos tratamentos ácido e térmico em paralelo, e então foram inoculadas em Agar BCYE com ou sem adição de antibióticos. Amostras não previamente tratadas foram também utilizadas. Seis amostras apresentaram um total de dezenove colônias positivas sendo onze confirmadas como *Legionella* sorogrupo 1, sete como *Legionella* sorogrupo 6 e uma como *Legionella bozemanii*. Melhores resultados foram obtidos nas amostras sem tratamento. Este trabalho comprova a presença de *Legionella* em nosso meio e a necessidade do desenvolvimento de métodos simples, rápidos e econômicos para sua detecção em programas de vigilância e controle.

Palavras-chave: *Legionella pneumophila, Legionella spp*, água.

REFERENCES

1. APHA. American Public Health Association. Standard Methods for the Examination of Water and Wastewater. 17ª ed. Washington, 1989, 149 p.
2. Barbaree, J.M., Fields, B.S., Feeley, J.C., Gorman, G.W., Martin, W.T. Isolation of protozoa from water associated with a Legionellosis outbreak and demonstration of intracelular multiplication of *Legionella pneumophila*. *Appl. Environ. Microbiol.* 51, 422-424, 1986.
3. Barbaree, J.M., Gorman, G.W., Martin, W.T., Fields, B.S., Morril, W.E. Protocol for sampling environmental sites for *Legionella*. *Appl. Environ. Microbiol.* 53, 1454-1458, 1987.
4. Bartlett, C.L.R., Kurtz, J.B., Hutchinson, J.G.P., Turner, G.C., Wright, A.E. *Legionella* in hospital and hotel water supplies. *Lancet* II: 1315-1317, 1983.
5. Brenner,D.J.; Feeley,J.C.; Weaver,R.E. Family VII. *Legionellaceae*. In:*Bergey's Manual Systematic Bacteriology*. 1: 279-288. Baltimore: William and Wilkins, 1984.
6. Cherry, W.B., Pittmam, B., Harris, P.P., Hebert, G.A., Thomosan, B.M., Thacker, L.; Weaver, R.E. Detection of Legionnaires' disease bacteria by direct immunofluorescent staining. *J. Clin. Microbiol.* 8: 329-338, 1979
7. Dutka, B.J. and Ewan, P. First isolation of *Legionella pneumophila* from the Canadian Great Lakes. *J. Great Lakes Res.* ,9(3):430-33, 1983.
8. Dutka, B.J., Walsh, K., Ewan, P., El-Shaarawi, A., Tobin, R.S. Incidence of *Legionella* organisms in selected Ontario (Canada) cities. *Scien. Total Environ. 39*: 237-249, 1984.
9. Dutka, B.J. and Ewan, P. Isolation of *Legionella pneumophila* from Canadian hot springs. *Can. J. Publ. Hlth.* 77: 136-138, 1986.
10. Edelstein, P.H., McKinney, R.M.; Meyer, R.D.; Edelstein, M.A., Krause, C.J., Finegold, S.M. Immunologic diagnosis of Legionnaires' disease: cross-reactions with anaerobic and microaerophilic organisms and infections caused by them. *J. Infect. Dis.* 141: 652-655, 1980.
11. Feeley, J., Gorman, G., Gibson, R. Primary isolation media and methods. IN: *Legionnaires' the disease, the bacterium and methodology* Ed. Jones, G.L. and Hebert,G.A. U.S. Dep. of Health, Education and Welfare. C.D.C. Atlanta, Georgia, 1979.
12. Fliermans, C.W., Cherry, W.B., Orrison, L.H., Smith, S.J, Tison, D.L., Pope, D.H. Ecological distribution of *Legionella pneumophila*. *Appl. Environ. Microbiol.* 41: 9 -16, 1981.
13. Fraser, D.W., McDade, J.E. Legionelose. *Investigation y Ciencia (Scientific American)* 39: 48-59, 1979.
14. Habitch, W., Muller, H.E. Ocurrence and parameters of frequency of *Legionella* in warm water systems of hospitals and hotels in Lower Saxony. *Zbl. Baki. Hyg. B.* 186:179-88, 1988.
15. Hussong, D., Colwell, R.R., O'Brien,M., Weiss,E., Pearson, A.D. Viable *Legionella pneumophila* not detectable by culture on agar media *Biotechn.* 5: 947-950, 1987.
16. Joly, J.R., Boissinot, M., Duchaine, J., Duval,M., Rafrari, J., Ramsan, D., Letarte, R. Ecological distribution of *Legionellaceae* in the Quebec city area. *Can. J. Microbiol.* 30: 63-67, 1983.
17. Joly, J.R., Dery, P., Gauvreau, L., Cote, L., Trepanier, C. Legionnaires' disease caused by *Legionella dumoffii* in destilled water. *Can. Med. Asso. J.*, 135(11): 1251-1252, 1986.
18. Lee, J.V. and West, A.A. Survival and growth of *Legionella* species in the environment. *J. Appl. Bact.* 70: 1219-1295, 1991.
19. Levin, A.S.S., Caiaffa Filho, H.H., Sinto, S.I., Sabbaga,E., Barone, A.A., Mendes, C.M.F. An outbreak of nosocomial Legionnaires' disease in a renal transplant unit in São Paulo, Brazil. *J. Hosp. Infect.*, 18: 243-248, 1991.
20. McDade, J.E., Shepard, C.C., Fraser, D.W., Tsai, T.R., Redys, M.A., Dowdle, W.R. Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *N. Engl. J. Med.* 297: 1197-1203, 1977.
21. McDade, J.E. Primary isolation usig guineapigs and embryonated eggs. IN: *Legionnaires' the disease, the bacterium and*

methodology. Ed. Jones, G.L. and Hebert, G.A. - U.S. Dep. of Health, Education and Welfare.C.D.C. Atlanta, Georgia, 1979.

22. McKiney, R.M., Thacker, L., Harris, P.P., Lewallen, K.R., Hebert, G.A., Edelstein, P.H., Thomason, B.M. Four serogroups of Legionnaires' disease bacteria defined by immunofluorescence. *Ann. Intern. Med.* 90: 621-624, 1979.
23. Mazzieri, N.A. De O. *Legionelose associada a pneumopatias em São Paulo - Estudo da comprovação etiológica por isolamento e sorologia.* 1990. Thesis for Master Degree. Instituto de Ciências Biomédicas, Universidade de São Paulo, Brasil.
24. Orrison, L.H., Bibb, W.F., Cherry, W.B., Thacker, L. Determination of antigenic relationships among legionellae and non-legionellae by direct fluorecent-antibody and immunodiffusion tests. *J. Clin. Microbiol.* 17: 332-337, 1983.
25. Ortiz-Roque, C.M. and Hazen, T.C. Abundance and distribuition of *Legionallaceae* in Puerto Rican waters. *Appl. Environ. Microbiol.* 53: 2231-2236, 1987.
26. Pereira Gomes, J.C., Mazzieri, N.A. De O., Godoy, C.V.F., Rocha, A.S. *Legionella pneumophila* associada a insuficiencia respiratoria aguda. Primeiro isolamento no Brasil. *Rev. Inst. Med. Trop. São Paulo* 31(6): 368-376, 1989.
27. Roberts, K.P., August, C.M., Nelson, J.D.J. Relative sensitivities of environmental *Legionellae* to selective isolation procedures. *Appl. Environ. Microbiol.* 53: 2704-2707, 1987.
28. Steele, T.W., Lanser, J., Sangster, N. Isolation of *Legionella longbeachae* serogroup 1 from potting mixes. *Appl. Environ. Microbiol.* 56: 49-53, 1990.
29. Stout, J.E., YU, V.L., BEST, M.G. Ecology of *Legionella pneumophila* within water distribution systems. *Appl. Environ. Microbiol.* 49: 221-228, 1985.
30. Tobin, R.S., Ewan, P., Walsh, K., Dutka, B.J. A survey of *Legionella pneumophila* in water in 12 canadian cities. *Water Res.* 20: 495-501, 1986.
31. Voss, L., Button, K.S., Tuovinen, O.H. *Legionella pneumophila* in a metropolitan water distribution system. *Environ. Tech. Letters,* 6: 429-438, 1985.
32. Wadowsky, R.M., Wilson, T.M., Kapp, N.J., West, A.J., Kuchta, J.M.; States, S.J., Dowling, J.N., Yee, R.B.. Multiplication of *Legionella* spp in tap water containing *Hartmannella vermiformis. Appl. Environ. Microbiol.* 57: 1950-1955, 1991.
33. Wilkinson, H.W., Fikes, .B.J., Farshy, C.E.. Indirect Immunofluorescense test for Legionnaires disease. *Legionnaires' the disease, the bacterium and methodology.* Jones, G.L. and Hebert, G. A. Ed. - U.S. Dep. of Health, Education and Welfare. C.D.C Atlanta, Georgia, 1979.
34. Wilkinson, I.J., Sangster, N., Ratcliff, R.M., Mugg, P.A., Davos, D.E., Lanser, J.A. Problems associated with identification of *Legionella* species from the environment and isolation of six possible new species. *Appl. Environ. Microbiol.* 56: 796-802, 1990.
35. Woo, A.H., Yu, V.L., Goetz, A. Potential in hospital modes of transmission of *Legionella pneumophila. Am. J. Med.* 80: 567-573, 1986.

Rev. Microbiol., São Paulo, **26**(3): 192-199, 1995

THIN-LAYER CHROMATOGRAPHY OF MYCOBACTINS AND MYCOLIC ACIDS FOR THE IDENTIFICATION OF CLINICAL MYCOBACTERIA

Clarice Q. F. Leite[1]
Angela M. W. Barreto[2]
Sergio R. A. Leite[3]

ABSTRACT

Forty seven strains of mycobacteria (35 strains isolated from clinical specimens and 12 reference strains) were analyzed for mycobactin and mycolate production by thin-layer chromatography (TLC). Different growth conditions had little or no effect on the production of individual mycobactins and the reproducibility of mycobactin Rf values. Mycolate profiles of isolated strains were compared with those of reference strains. Clinical isolates belonging to the same species showed the same profiles. The combined evaluation of mycobacterial products by TLC allowed the identification of pathogenic and opportunist cultivable mycobacteria. On routine examination, the analysis of mycobactin and mycolate production constitutes an adequate procedure for the characterization and identification of mycobacteria.

Key words: mycobacteria, identification, mycobactin, mycolic acid, thin layer chromatography.

INTRODUCTION

The identification of species of Mycobacteria by morphological, biochemical and culture tests requires several weeks or even months of fastidious work[5]. So, faster identification techniques are desirable to allow an earlier specific treatment of mycobacteriosis. The utilization of different chemical methods has greatly improved the classification and identification of mycobacteria[2,3,4,9,11]. Snow and White[13] suggested an approach useful for direct identification of the species involved, by evidencing detectable variations in the chemical composition of mycobactins. These are lipid-soluble compounds able to chelate iron and produced by mycobacteria under iron-deficient growth conditions. Species-specific differences between mycobacterial species could be readily observed by means of thin-layer chromatography (TLC) of the produced mycobactins. Hall and Ratledge[6] developed a simple culture medium which provided good mycobactin accumulation. They also proposed a rapid method of extraction of mycobactins and their further identification by TLC[6]. The composition of mycobacterial lipids has also been used to separate

[1] Depto. de Ciências Biológicas, Faculdade de Ciências Farmacêuticas - UNESP Araraquara - SP
[2] Centro de Referência "Prof. Helio Fraga", Rio de Janeiro - RJ
[3] Depto. de Química Geral e Inorgânica, Instituto de Química - UNESP - Araraquara - SP
Correspondence, proofs and requests of reprints to:
Clarice Queico Fujimura Leite. Departamento de Ciências Biológicas - Faculdade de Ciências Farmacêuticas - UNESP . Telefone: (016)232.0200 Ramal 291. FAX: 016-232.1576. Rodovia Araraquara-Jaú, km 1. 14801-902 - Araraquara - SP , Brasil.

distinct mycobacterial species[8,12,14]. Some reports show the determinations of mycolic acid patterns by TLC and recommend them as a first step for routine identification of mycobacteria in the clinical laboratory [9,10,11].

TLC is a rapid and simple technique for mycobactin and mycolic acid identification, although it is not utilized by Brazilian reference laboratories. Standard procedures must be developed before using this method in a larger scale. The purpose of this study was to evaluate the feasibility of using TLC for mycobactin and mycolic acid analysis during routine identification of clinically isolated mycobacteria in Brazil.

MATERIALS AND METHODS

Bacterial strains

The sources of the 47 strains selected for this study are listed below. Twelve reference strains were obtained from the Collection Institut Pasteur Tuberculose (CIPT), Paris, while 35 strains isolated from clinical specimens were provided by Campanha Nacional Contra a Tuberculose (CNCT), Rio de Janeiro. They were maintained on Lowestein-Jensen (LJ) medium slants at refrigeration temperature. For each strain, three parallel experiments were performed .

Reference Strains

M. avium-intracellulare CIP 140310001, *M. bovis* CIP 140020001,*M. chelonae*CIP 140420003, *M. fortuitum* CIP 140410001, *M. gordonae* CIP 140210001, *M. kansasii* CIPT 140110001, *M. marinum* CIP 140120001, *M. nonchromogenicum* CIP 141030004, *M. scrofulaceum* CIP 140220001, *M. smegmatis* CIP 141330100, *M. szulgai* CIP 140240001 and *M. tuberculosis* CIP 140010002.

Mycobacteria isolated from clinical specimens (CNTC):

Eleven *M. avium-intracellulare,* 2 *M. bovis* (BCG), 3 *M. chelonae*, 6 *M. fortuitum*, 4 *M. gordonae,* 3 *M. kansasii,* 2 *M. scrofulaceum*, 1 *M. szulgai* and 3 *M. tuberculosis.*

Media and growth conditions

For mycobactin formation, mycobacteria were initially grown in a simple liquid glycerol-asparagine-mineral salts medium (GAM[6]) and in GAM modified medium (GAM-M) containing dextrose in place of glycerol and 0,8% Nutrient Broth (Difco). Incubation temperatures were 30°C for *M. marinum* and *M. chelonae* and 35°C for the other mycobacteria. The incubation period ranged from 7 to 10 days for rapidly growing species, and from 25 to 60 days for slow growing species. Samples of 0,5mL were removed from each culture and inoculated onto GAM or GAM-M solidified with 2% Bacto Agar (Difco), in order to promote mycobactin formation. These cultures were then incubated under the same conditions described above until a good growth was achieved. For the production of mycolic acids, all strains were grown on LJ slants and incubated as already stated.

Preparation of samples

Mycobactins were extracted with ethanol from mycobacterial cells according to the method of Hall and Ratledge[6]. $FeCl_3$ in ethanol was added to the extract, until no further change in color occurred (the solution turns to reddish amber due to formation of ferric mycobactin). An equal volume of chloroform was added, followed by water until two layers were formed. The chloroform phase was separated and shaken three times with aqueous sodium sulfate solution to remove excess iron (pure water forms a persistent emulsion). It was then dried with anhydrous sodium or magnesium sulfate and evaporated to dryness by heating under vacuum.

Mycolic acid analysis was carried out according to the method of Daffe *et al.*[4] with some modifications. Approximately 25-50 mg of mycobacteria derived from Lowenstein-Jensen medium were dispersed into a 5% potassium hydroxide solution in 2-methoxyethanol. The mixture was warmed at 110°C for 2 h, cooled and acidified with 1 mL of 20% (w/w) sulfuric acid in water. This method saponifies mycobacterial lipids, and mycolic acids are released from their potassium salts by the sulfuric acid treatment. The mycolic acids were then extracted shaking the mixture two times with diethyl ether (5 mL). The ether phase was decanted and washed three times with 5 mL of

water. Ether was evaporated on a water bath, leaving a residue of mycolic acids that were methylated by addition of 1 mL of diazomethane ether solution. This reagent was prepared by reacting nitrosomethylurea (synthesized in our laboratory) with potassium hydroxide.

Analytical methods

TLC analysis of the mycobactins was always performed in a single dimension, employing ether solution of mycobactin spotted onto 20 x 20 cm plates, with layer thickness of 0.25 mm. Two systems of adsorbents and solvents were employed. System I: silica gel G (Merck), which was developed with petroleum ether (b.p. 65 to 110°C) / diethyl acetate / n-butanol (2:3:3, by vol.). System II: aluminum oxide 60 F_{254} (Merck) with cyclohexane / n-butanol (9:1, v/v)[6].

For TLC of mycolic acids, their methyl esters were spotted onto silica gel G (20 x 20cm x 0.25 mm). One dimensional analysis was carried out using two different elution systems: diethyl ether / petroleum ether (12:88 V/V), with three developments of the chromatogram, and dichlorometane with only one development[10]. The presence of the separated components was revealed by spraying the chromatograms with 0,01% (w/v) rhodamine in phosphate buffer[5].

Identification of mycobactins and mycolic acids

Spots of mycobactins were identified by determination of Rf values. The mycolic acid profiles of clinical isolates were compared with standard patterns from reference mycobacteria.

RESULTS AND DISCUSSION

Mycobactin analysis

The production of mycobactins was evaluated by comparing data from GAM and GAM-M based cultures; the reproducibility of Rf values was verified by system I chromatography. Reference strains were grown under iron-deficient conditions on GAM and GAM-M solid media (three parallel experiments) and their mycobactins extracted and analyzed by TLC. In the case of *M. scrofulaceum*, no mycobactin production was observed on both media. With respect to the other mycobacteria, GAM-M was found to be a better culture medium, since it allowed mycobactin production even with strains that had failed to yield detectable mycobactin concentrations in GAM, *M. gordonae* being an exception. The Rf values for the mycobactins obtained in either GAM or GAM-M were comparable. This indicates that the mycobactins are quite similar in chemical structure, even when using a modified culture medium richer than the original one, which permits the growth of more fastidious mycobacteria. Barclay *et al.*[1] had similar problems when working with slow growth bacteria in GAM medium. Bosne and Lévy-Frébault[2], in order to stimulate mycobacterial growth, proposed the use of a rich liquid medium containing an iron immobilizing chelator (ethylenediamine-di-o-hydroxyphenylacetic acid) to ensure iron starvation. TLC of mycobactins from reference mycobacteria by systems I and II are shown in FIGS. 1 and 2.

The chromatography of mycobactins using two systems was necessary because different mycobactins may have the same Rf for a given chromatographic procedure. For example, mycobactins of *M. avium-intracelulare* and *M. smegmatis* have almost the same Rf when applying system I, but are distinguishable by system II. This is also the case of the assemblage *M. gordonae*, *M. nonchromogenicum* and *M. szulgai*, where the species are not differentiated by system I but can be resolved by system II. Conversely, the mycobactins of *M. gordonae* and *M. tuberculosis* are not separated by system II but can be singled out by system I.

Mycolic acids analysis

The mycolic acid profiles of reference strains are shown in TABLE 1 and in FIGS. 3 and 4. The results are in agreement with previously reported data[5,12].

Profiles composed of ∝-mycolate (type I), methoxymycolate (type III) and ketomycolate (type IV) were found for *M. tuberculosis*, *M. bovis*, *M. marinum*, *M. kansasii*, *M. szulgay* and *M. gordonae*. Profiles composed of ∝-mycolate, ketomycolate and carboxymycolate (type VI) were found for *M. avium-intracellulare*, *M. scrofulaceum* and *M. nonchromogenicum*. *M. fortuitum* and *M.*

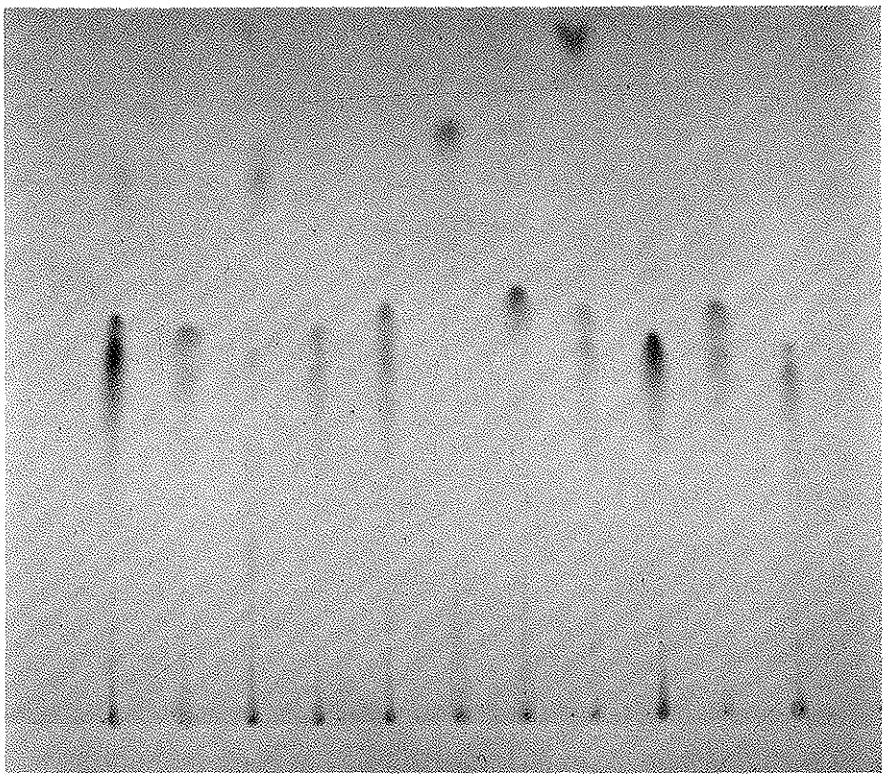


FIGURE 1: Chromatogram of mycobactins from reference strains of mycobacteria. System I: adsorvent - silica gel 60G (Merck); solvent - petroleum ether/ethylacetate/n-butanol (2:3:3 U/V).
Reference strains, from left to right: *M. avium-intracellulare, M. bovis, M. chelonae, M. fortuitum, M. gordonae, M. kansasii, M. marinum, M. nonchromogenicum, M. smegmatis, M. szulgai, M. tuberculosis.*

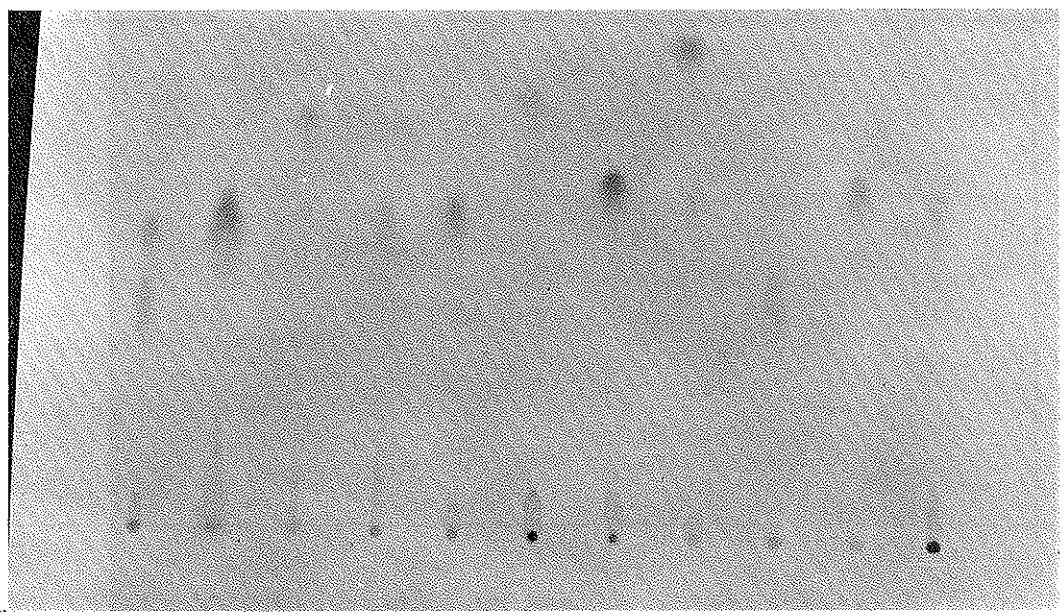


FIGURE 2: Chromatogram of mycobactins from reference strains of mycobacteria. System II: adsorvent - aluminum oxide $60F_{254}$ (Merck); solvent - cyclohexane/n-butanol (9:1 U/V).
Reference strains, from left to right: *M. avium-intracellulare, M. bovis, M. chelonae, M. fortuitum, M. gordonae, M. kansasii, M. marinum, M. nonchromogenicum, M. smegmatis, M. szulgai, M. tuberculosis.*

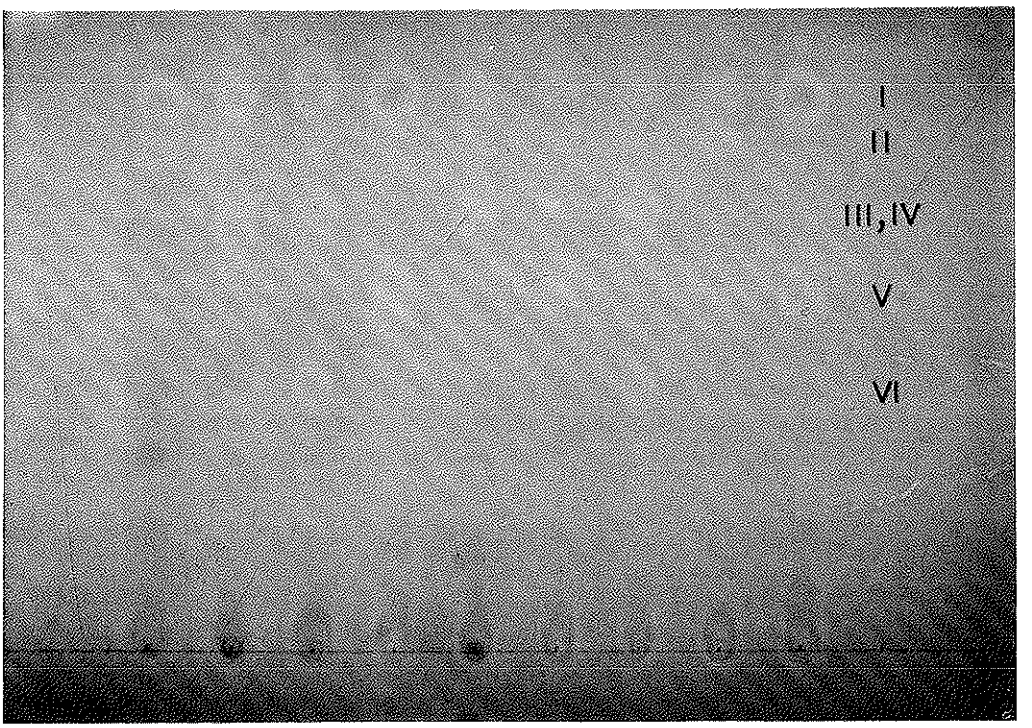


FIGURE 3: Chromatogram of mycolic acid methyl esters. Solvent: diethyl ether - petroleum ether.
Reference strains of micobacteria, from left to right: *M. chelonae* (I.II), *M. tuberculosis* (I.III.IV), *M. avium-intracellulare* (I.IV.VI), *M. fortuitum* (I.V), *M. bovis* (I.(III).IV), *M. smegmatis* (I.(II).V), *M. scrofulaceum* (I.IV.VI), *M. chelonae* (I.II), *M. fortuitum* (I.V).

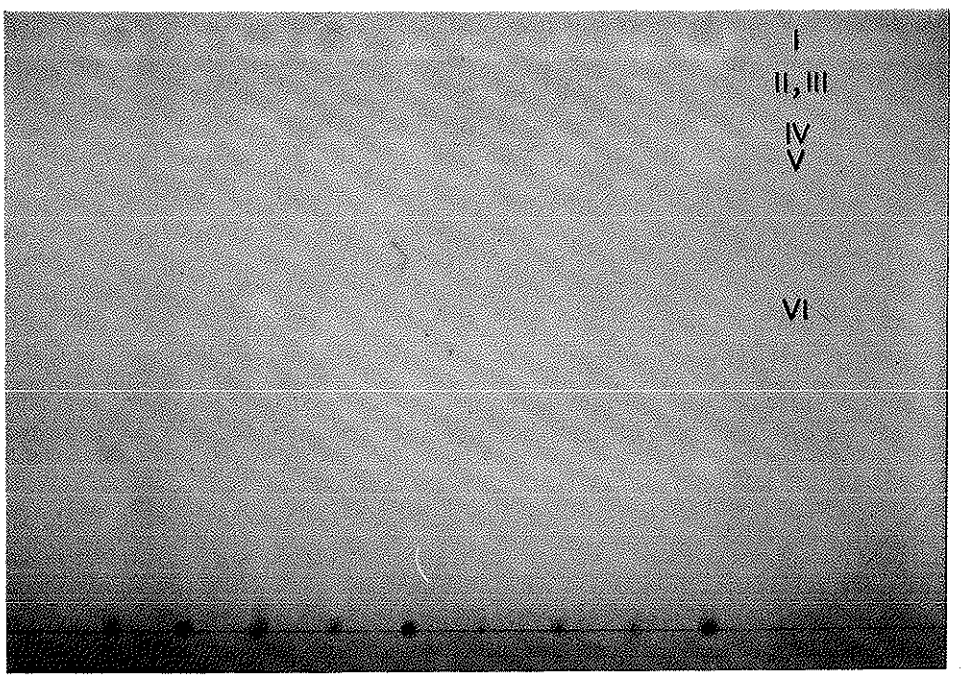


FIGURE 4: Chromatogram of mycolic acid methyl esters chromatogram. Solvent: dichloromethane
Reference strains of mycobacteria, from left to right: *M. chelonae* (I.II), *M. tuberculosis* (I, III, IV), *M. avium-intracellulare* (I.IV.VI), *M. fortuitum* (I.V), *M. bovis* (I.(III).IV), *M. smegmatis* (I (II) V), *M. scrofulaceum* (I.IV.VI), *M. chelonae* (I, II), *M. fortuitum* (I, V)

smegmatis contained ∝-mycolate and epoxymycolate (type V). *M. chelonae* had a profile of ∝ and ∝-mycolate (type II). Since an accurate differentiation between mycolate types II and III seems to be necessary, two developing systems were used[4]. Mycolates II and III showed similar Rf values in diethyl ether-petroleum ether solvent, and both migrated just below mycolate I. However, in dichloromethane, mycolate III migrated below mycolate II and its Rf value was similar to that of mycolate IV (FIGS. 2 and 3).

Correlation between mycobactins Rf values and mycolate profiles

The correlation between Rf profiles of mycobactins and mycolic acids for the studied mycobacteria is presented in TABLE 1.

M. tuberculosis, *M. bovis*, *M. marinum*, *M. kansasi*, *M. szulgai* and *M. gordonae* show the same mycolate profiles. However, these mycobacteria may be distinguished by their corresponding mycobactin Rf profiles obtained with systems I and II. The first 4 mycobacteria may be differentiated by chromatograms from system I. *M. szulgai* and *M. gordonae* have the same Rfs using system I, which have different resolutions using system II. Likewise, it is possible to differentiate the strains of the MAIS Complex (*M. avium-intracellulare* - *M scrofulaceum*) from *M. nonchromogenicum*, all of which contain mycolic acids I, IV and VI, by evaluation of mycobactin Rf profiles (system II). Within the MAIS Complex, *M. scrofulaceum* does not produce enough mycobactin for analysis, whereas *M. avium-intracelulare* produces detectable amounts. Only strains that belong to the MAIS Complex are considered potentially pathogenic mycobacteria, and their differentiation is thus very important.

TABLE 1. Reference strains of mycobacteria: mycobactin Rf values (systems I and II) and mycolate profiles.

Mycobacterium	Mycobactin Rf values system I	system II	Mycolate profiles
M. avium-intracellulare	0,47	0,28 0,38	I.IV.VI
M. bovis	0,51	0,4	I.(III)*.IV
M. chelonae	0,71	0,53	I.II
M. fortuitum	0,50	0,40	I.V
M. gordonae	0,53	0,42	I.III.IV
M. kansasii	0,78	0,57	I.III.IV
M. marinum	0,56	0,46	I.III.IV
M. nonchromogenicum	0,53	0,43 0,65	I.VII
M. scrofulaceum	-	-	I.IV.VI
M. smegmatis	0,49	0,33	I.(II)*.V
M. szulgai	0,54	0,47	I.(III)*.IV
M. tuberculosis	0,45	0,43	I.III.IV

* Trace amounts

M. fortuitum and *M. smegmatis*, both with mycolates I and V, is easily performed by mycobactin analysis with system II (they present the same Rf values with system I). This result can be confirmed by the arylsulfatase test[5]. *M. fortuitum* is also an opportunistic mycobacterium while *M. smegmatis* is a saprophyte. *M. chelonae* has a mycolic acid profile different from that of the other mycobacteria, a feature that makes its identification very simple.

FIGURE 4 shows a theoretical system proposed for mycobacterial identification.

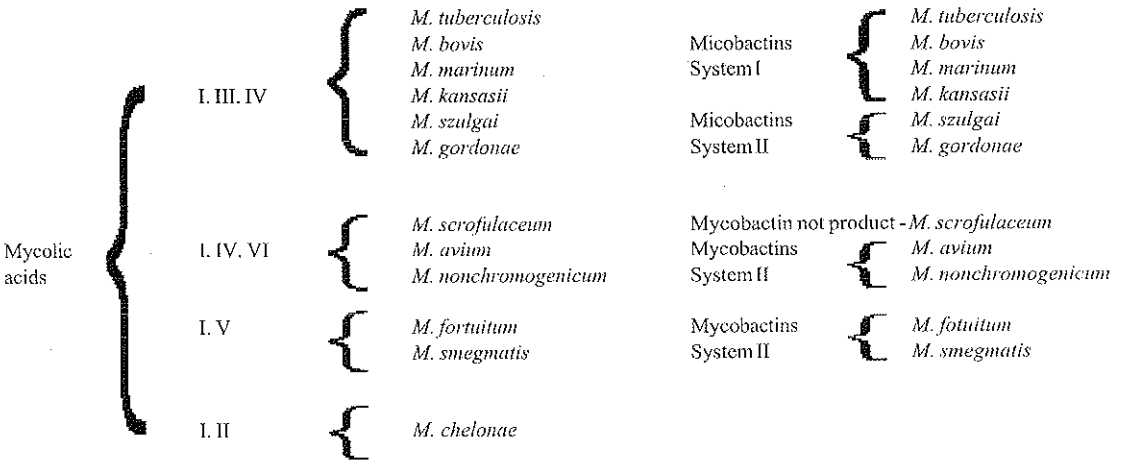
Identification of clinically isolated mycobacteria by TLC

The 35 mycobacteria strains isolated from clinical specimens and identified by classical procedures were tested by the presently proposed method, associating mycobactins and mycolic acids profiles. For all the strains, the results obtained by TLC corroborated the characterizations made with the traditional methods.

CONCLUSION

Mycobactin and mycolic acid analyses, performed as separate or combined techniques, were shown to be a valuable tool for mycobacterial identification. The simultaneous use of mycobactin and mycolate analyses by TLC permitted the identification of all the reference strains studied. Under the reported conditions, the use of TLC for evaluation of mycobactins and mycolates may be considered a rapid and reproducible method, recommendable as a simple routine clinical test for the identification of mycobacteria in reference laboratories.

FIGURE 5: - Theoretical system for the identification of mycobacteria.



ACKNOWLEDGMENTS

This work was supported by FAPESP (grant n° 89/3474-2).

We thank Dr. Petr Melnikov for correcting the English language of the manuscript.

RESUMO

Identificação de micobactérias de interesse clínico por cromatografia em camada delgada de micobactina e de ácidos micólicos

Quarenta e sete amostras de micobactérias (12 de referência e 35 isolados de espécimes clínicos) foram analisadas quanto à produção de micobactinas e ácidos micólicos, pela técnica de cromatografia em camada delgada (CCD). Diferentes condições de crescimento pouco ou nada afetaram o perfil de separação das micobactinas. Foi verificada a reprodutibilidade nos valores de Rf das micobactinas das diferentes espécies analisadas. Os perfis de ácidos micólicos foram comparados àqueles de amostras padrões. Todas as amostras da mesma espécie, isoladas de espécimes clínicos, mostraram os mesmos perfis. O uso combinado dos 2 métodos químicos permitiu a identificação das micobactérias patogênicas ou oportunistas cultiváveis. Para o exame de rotina, a análise de micobactinas e de micolatos provê um meio simples e adequado de caracterização e identificação de micobactérias.

Palavras-chave: micobacteria, identificação, micobactina, ácido micólico, cromatografia em camada delgada.

REFERENCES

1. Barclay, R.; Ewing, D.F.; Ratledge, C. Isolation, Identification, and Structural Analysis of the Mycobactins of *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium scrofulaceum*, and *Mycobacterium partuberculosis*. *J. Bacteriol.*, 164: 896-903, 1985.
2. Bosne, S.; Lévy-Frébault, V.V. Mycobactin Analysis as an Aid for the Identification of *Mycobacterium fortuitum* and *Mycobacterium chelonae* subspécies. *J. Clin. Microbiol.*, 30: 1225-1231, 1992.
3. Butler, W.R.; Kilburn, J.O. High-Performance Liquid Chromatography Patterns of Mycolic Acids as Criteria for Identification of *Mycobacterium chelonae, Mycobacterium fortuitum*, and *Mycobacterium smegmatis*. *J. Clin. Microbiol.*, 28: 2094-8, 1990.
4. Daffé, M.; Lanéelle, M.A.; Asselineau, C.; Lévy-Frébault, V.; David, H. Intéret taxonomique des acides gras des mycobactéries: proposition d'une méthode d'analyse. *Ann. Microbiol. (Inst. Pasteur)* 134B: 241-256, 1983.
5. David, H.L.; Lévy-Frébualt, V.; Throrel, M.F. *Méthodes de laboratoire pour mycobacteriologie clinique*. França, Unité de la Tuberculose et des Mycobacteries, Institut Pasteur, 1989. p.58-63.
6. Hall, R.M.; Ratledge, C. A simple method for the production of mycobactin, the lipid-soluble siderophore, from mycobacteria. *FEMS. Microb. Letters*, 15: 133-136, 1982.
7. Hines, M.E.; Frazier, K.S. Differentiation of Mycobacteria on the basis of chemotype profiles by using matrix solid-phase

dispersion acid thin-layer chromatography. *J. Clin. Microbiol.*, 31: 610-614, 1993.

8. Leite, C.Q.F.; Giannini, M.J.S.; Lévy-Frébault, V.; David, H.; Falcão, D.P Presence of *Mycobacterium marinum* and other oportunist mycobacteria in swimming pool waters in Araraquara, SP. *Rev. Microbiol.*, 19: 354-359, 1988.
9. Leite, C.Q.F.; Ferracini Jr, R.; Falcão, D.P.; David, H.; Lévy-Frébault, V. Prevalência e distribuição de micobactérias nas águas de algumas regiões do Estado de São Paulo - Brasil. *Rev. Microbiol.*, 20: 432-411, 1989.
10. Lévy-Frébault, V J.; Goh, K.S.; David, H.L. Micolic acid analysis for clinical identification of *Mycobacterium avium* and related mycobacteria. *J. Clin. Microbiol.*, 24: 835-839, 1986.
11. Luquin, M.; Ausina, V.; Calahorra, F.L.; Belda, F.; Barceló, M.G.; Celma, C.; Prats, G. Evaluation of Practical Chromatographic Procedures for Identification of Clinical of Mycobacteria. *J. Clin. Microbiol.*, 29: 120-30, 1991.
12. Minnikini, D.E.; Minnikini, S.M.; Parlett, J.H.; Goodfellow, M.; Magnusson, M. Mycolic acid pattern of some species of *Mycobacterium*. *Arch. Microbiol.*, 139: 225-231, 1984.
13. Snow, G.A.; White, A.J. Clinical and biological properties of mycobactins isolated from various mycobacteria. *Biochem. J.*, 115: 1031-45, 1969.
14. Thibert, L.; Lapierre, S. Routine application of hight-performance liquid chromatography for identification of Mycobacteria. *J. Clin. Microbiol.*, 31: 1759-1763, 1993.

Rev. Microbiol., São Paulo, **26**(3): 200-202, 1995

FIRST-VOID VERSUS MIDSTREAM URINE CULTURE FOR DETECTION OF *UREAPLASMA UREALYTICUM* IN THE URINARY TRACT

Maria Stella G. Raddi[1*]
Nancy C. Lorencetti[1]
Silvia A. Rodrigues[2]

ABSTRACT

Quantitative *Ureaplasma urealyticum* culture was performed on clean first-void and midstream urine to evaluate the presence of these mollicutes in the urinary tract. The results, expressed as color changing units (CCU), showed that 14 (63%) of the 22 *Ureaplasma urealyticum* positive patients yielded counts equal to or higher than 10^7 CCU/mL for both the initial and the middle urine specimens. No abnormal chemical or microscopic findings (protein content, leukocyte numbers) were observed. The occurrence of *U. urealyticum* in midstream urine samples, even when numbers are considered, may be no more than a guide to the presence of ureaplasmas in the urinary tract.

Key words: *Ureaplasma urealyticum*, urine culture, urinary tract infection.

INTRODUCTION

Since ureaplasmas were first isolated by Shepard in 1954, there has been an increasing interest in mycoplasmas as causative agents of urogenital tract infection. Some investigators have isolated *Ureaplasma urealyticum* more frequently from patients suffering from nongonoccocal urethritis (NGU) than from apparently healthy controls, whereas other authors report no significant differences in the rate of isolation between these two groups of subjects (6).

Most urinary tract infections result from ascending infection by organisms introduced through the urethra. Infections are usually associated with bacterial counts of 100.000 (10^5) or more organisms per mL of urine. In contrast, contamination from the external genitalia in the absence of infection usually yields less than 1.000 (10^3) organisms per mL in properly collected and transported specimens.

U. urealyticum has been isolated from the bladder and upper urinary tract, being recovered more often from patients with reflux nephropathy than from patients with other renal diseases. This suggests that ureaplasmal colonization contributes to progressive renal disease (7).

In this study, we compared ureaplasmal counts of first-void and clean midstream urine from patients without signs of urinary tract disease.

MATERIALS AND METHODS

Clean first-void and midstream early morning urine specimens were obtained from 47

[1] Department of Clinical Microbiology (MD), University Júlio de Mesquita Filho/ UNESP, Araraquara, Brazil.
[2] Research Assistant supported by grant from FUNDAP.
[*] To whom correspondence should be sent, at the address: Faculdade de Ciências Farmacêuticas, Rodovia Araraquara-Jaú - Km 1 - UNESP CP 502 14.801-902 Araraquara, SP, Brazil. Phone: 55(162)321233, branch 146; Fax: 55(162)321576.

asymptomatic patients. Viable ureaplasmas were quantified as color changing units (CCU) using the tube dilution method described by Rodwell and Whitcomb(3). When a visible change in the indicator occurred, a 0,1mL sample was subcultured on agar medium and in a liquid medium. *U. urealyticum* was identified by its colony size and morphology, urease production and oxidation of manganese salt on ureaplasmal agar. The estimated numbers of CCU were determined by the highest dilution at which ureaplasma was detected.

Routine urinalysis was performed on all first urine samples using Ames Multistix, recording protein content, presence of blood, specific gravity and pH; trace amounts of protein and glucose were not considered positive results. Ten mL of each sample were centrifuged for 5 min at 1,500 r.p.m., and 9 mL of the supernatant were then discarded. The sediment was resuspended in the remaining 1 mL with a Pasteur pipette. Leukocyte numbers were determined in a Tiefe Neubauer counting chamber.

RESULTS

U. urealyticum was recovered from both first-void and midstream urine in 22 (47%) of 47 patients. The sex and age distribution for the different groups of patients is summarized in TABLE 1. A positive result in first-void urine only was recorded in a single case. TABLE 2 compares *Ureaplasma urealyticum* positive results for first-void and midstream urine according to CCU. There were no abnormalities on the biochemical and microscopic findings for all the specimens. No significant correlation between leukocyturia and high titles of *U. urealyticum* was observed. The median counts for first-void and midstream urine in the group of subjects with negative culture were 8,768 and 3,820 cells/mL respectively, whereas the corresponding counts for individuals with positive ureaplasma culture were 7,777 and 4,359 cells/mL.

DISCUSSION

A causal relationship between ureaplasmal colonization and urogenital disease has not been convincingly demonstrated. The difficulty is partly due to the fact that *Ureaplasma urealyticum* can be found in the urethral flora of asymptomatic individuals. One of the reasons why some studies have failed to establish this relationship is that they have been qualitative rather than quantitative, not documenting the numbers of organisms isolated from individual patients. If ureaplasmas are involved in the pathogenic process during infection, it is reasonable to reason that in such situations their numbers would be much greater than the counts expected if they had only a commensal role. In this respect, some studies have previously suggested that quantitative cultures for ureaplasma might help to elucidate the pathogenic role of *U. urealyticum* in NGU. Our quantitative results, expressed as color-changing units, showed concentrations of ureaplasmas for first-void urine equal or greater than those for midstream urine. There were no differences in leukocyte numbers when comparing *U. urealyticum* positive and negative groups.

Ureaplasmas have been recovered by

TABLE 1. Sex and age distribution among patients with and without *Ureaplasma urealyticum* in urine

	U. urealyticum		Total
	with	without	
Sex			
N(%) of female	18 (46)	24 (54)	39
N(%) of male	4 (50)	4 (50)	8
Age			
N(%) ≤20 yr old	2 (50)	2 (50)	4
N(%) 21-39 yr old	17 (49)	18 (51)	35
N(%) ≥40 yr old	3 (30)	5 (62)	8

TABLE 2. Comparison of *Ureaplasma urealyticum* counts (CCU/mL) in first-void and midstream urine

No of patients	*U. urealyticum* (CCU/mL)	
	fist-void	midstream
14	$\geq 10^7$	$\geq 10^7$
3	$\geq 10^7$	10^6
3	$\geq 10^7$	10^5
1	10^6	10^6
1	10^3	

CCU - color changing units

suprapubic puncture from patients with bladder disease. It has been demonstrated that they induce crystallization of struvite and calcium phosphate in artificial urine *in vitro,* and also that they produce calculi in animals models (5). Thomsen (7) demonstrated the presence of *U. urealyticum* together with other bacteria in upper urinary tract specimens (taken during surgery from opened renal pelvis) of patients with acute pyelonephritis. This may indicate that the role of *U. urealyticum* in the upper urinary tract is secondary to bacteria. Recent carefully controlled clinical studies have suggested that only in a subpopulation of individuals with lower genitourinary tract infection does the organisms reach the upper tract, and that, furthermore, only in some of these individuals does disease ensue. In no instance the factors that predispose to upper tract colonization or disease development have been defined (1).

The recognition of 14 serotypes within the *Ureaplasma urealyticum* species has led to attempts to link ureaplasmal pathogenicity with particular serotypes. Shepard and Lunceford (4) found that serotype IV was predominant among several groups of patients, including those with NGU. Hewish *et al* (2), on the other hand, found no association between urinary tract disease and serotypes. The existence of virulent and avirulent strains has been proposed to account for this anomaly.

From the results obtained in this study it seems reasonable to conclude that the urinary tract may become invaded with ureaplasmas, and the source of contamination of midstream urine can be assumed to be the lower urethra. *U. urealyticum* in midstream urine samples, even when numbers are considered, may be no more than a guide to the presence of ureaplasmas in the urinary tract. Further studies will be necessary to determine the precise role of these mollicutes, if any, in disease of the renal system of humans.

RESUMO

Urina de primeiro jato *versus* jato médio para detecção de *Ureaplasma urealyticum* no trato urinário

Cultura quantitativa para *Ureaplasma urealyticum* foi realizada a partir de urina colhida, após antissepsia, de primeiro jato e jato médio com o objetivo de detectar a presença desse molicute no trato urinário. Os resultados, expressos em CCU (color changing units), mostraram que 14 (63%) dos 22 pacientes com cultura positiva albergavam o microrganismo em quantidades iguais ou superiores a 10^7 CCU/mL nas porções de urina obtidas de primeiro jato e jato médio. Não foram observadas alterações químicas ou microscópicas (proteínas, leucócitos) nos materiais examinados. *U. urealyticum* em amostra de urina colhida de jato médio, mesmo em quantidade considerável, não elucida a patogenicidade desse microrganismo.

Palavras-chave: *Ureaplasma urealyticum*, cultura de urina, infecção do trato urinário.

REFERENCES

1. Cassell, G. H.; Waites, K. B.; Taylor-Robinson, D. Genital mycoplasmas. *In*: Morse, S.A.; Moreland, A.A. and Thompson, S.E. (eds). *Atlas of sexually transmitted diseases*, Harper and Row Ltda, New York, 1990.
2. Hewish, M.; Birch, D.F.; Fairley, K.F. *Ureaplasma urealyticum* serotypes in urinary tract disease. *J. Clin. Microbiol.*; 23:149-54. 1986.
3. Rodwell, A.W.; Whitcomb, R.C. Methods for direct and indirect measurement of mycoplasma growth. *In*: Razin, S.; Tully, J.G. (eds). *Methods in mycoplasmology. London. Academic Press, Inc.* Vol II. 1983.
4. Shepard, M. C.; Lunceford, C.D. Serological typing of *Ureaplasma urealyticum* isolates from urethritis patients by an agar growth inhibition method. *J. Clin. Microbiol.*; 8:566-74. 1978
5. Tabeke, S.; Numata, A.; Kobashi, K. Stone formation by *Ureaplasma urealyticum* in human urine and its prevention by urease inhibitors. *J. Clin. Microbiol.*; 20:869-73. 1984
6. Taylor-Robinson, D. Mycoplasmal and mixed infections of the human male urogenital tract and their possible complications. *In*: Razin, S.; Barile, M.F. (eds). *The mycoplasmas. Florida. Academic Press, Inc.* Vol IV. 1985.
7. Thomsen, A.C. Occurrence of mycoplasmas in urinary tracts of patients with acute pyelonephritis. *J. Clin. Microbiol.*; 8:84-8. 1978.

Rev. Microbiol., São Paulo, **26**(3): 203-210, 1995

STABILITY OF A RECOMBINANT PLASMID IN *ZYMOMONAS MOBILIS* AG11[1]

Newton Portilho Carneiro [2]
Walter Vieira Guimaraes [2*]
Elza Fernandes de Araújo [2]
Arnaldo Chaer Borges [2]

ABSTRACT

Nineteen recombinant plasmids containing fragments of *Zymomonas mobilis* Ag11 plasmidial DNA cloned at the EcoRI site of vector pBR325 were obtained. The cloned fragments ranged from <0.5 to 8.6 kb. The weight of the recombinant plasmid pZMB12 was increased by recombination processes during its permanence in *Zymomonas mobilis* Ag11. Upon EcoRI cleavage, this plasmid, denoted pZBMA12, showed that pBR325 had maintained its original size. The recombinant plasmid pZMB12 showed greater stability in *Zymomonas mobilis* than in *E. coli*.

Key Words: *Zymomonas mobilis*, recombinant plasmid.

INTRODUCTION

Zymomonas mobilis is a facultative anaerobic bacterium capable of fermenting glucose, fructose and sucrose with the production of ethanol (33). Due to its resistance to high ethanol and sugar concentrations and ability to grow at low medium pH media and independently of ethanol production, this organism has the potential for application in the industrial production of ethanol (15, 26). Recombinant DNA techniques may permit the expansion of substrates that can be fermented by *Zymomonas mobilis* (27), with particular attention paid to lactose, starch and cellulose. The transfer of the Lac-operon from enteric bacteria (6, 9) and of transposon Tn 951 containing the Lac-operon to *Z. mobilis* (7, 32) resulted in transformants with a low level of ß galactosidase expression. The genes of *Cellulomonas uda* involved in the utilization of cellulose were expressed in *Z. mobilis* under the control of the promoter of the chloramphenicol transferase gene (23), but the cellulase complex was not excreted into the culture medium. The genetic manipulation of *Z. mobilis* is mainly impaired by its efficient restriction system, which affects the establishment and expression of exogenous genes (25).

Different natural plasmids have been detected and characterized in *Z. mobilis* (11, 13, 29, 31), and some are used in the construction of cloning vectors for this bacterium (1, 8, 22, 34). The use of plasmid DNA fragments of *Z. mobilis* is a strategy aiming at the stabilization of these vectors.

In the present study, a recombinant plasmid was constructed using plasmidial DNA segments of *Z. mobilis* Ag11 inserted into the EcoRI site of vector pBR325.

---

[1] Part of the M.Sc. thesis of the first author

[2] Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, M.G., Brasil - 36570-000

[*] To whom correspondence should be addressed

Table 1 - Relevant characteristics of the bacterial strains and plasmids.

Strains	Plasmids	Relevant Characteristics	References
E. coli(HB101)	-	F^-, $RecA^-$, Str^R	5
E. coli(DH5α)	-	F^-, $RecA^-$, r^-, Str^S	2; 17
Z. mobilis (Ag11)	-	Nal^R, Str^R, Kam^R, Tet^S	19
E. coli(C600)	pBR325	Ap^R, Cm^R, Tc^R	4
E. coli(MM294)	pRK2013	Km^R, Tra'	16

MATERIALS AND METHODS

The bacterial strains and plasmids and their relevant characteristics are indicated in TABLE 1. *E. coli* was grown in LB culture medium (21) and *Z. mobilis* in RM medium (30).

The natural plasmids of *Z. mobilis* Ag11 were extracted by the procedures of Birnboim and Doly (3) and Maniatis *et al.* (20). Plasmid DNA was analyzed on 0.8% agarose gel in a horizontal cuvette by applying a 35 mA current. Agarose was dissolved in 89 mM TEB buffer, pH 8.0. For DNA visualization ethidium bromide was used at a final concentration of 0.5 μg/mL mixed with agarose. The gels were analyzed in a UV transilluminator and photographed with an Asahi-Pentax camera attached with an orange-colored filter and a Kodak Plus-X Pan-PXKO2 film.

The size of plasmid DNA fragments cloned in pBR325 was determined by the method of Schaffer and Sederoff (28) with the program developed at the Center of Biotechnology, Federal University of Rio Grande do Sul, employing fragments of lambda CI857 DNA cleaved with HindIII.

The preparation of plasmid DNA of *Z. mobilis* Ag11 was cleaved with the restriction enzyme EcoRI and the resulting fragments were bound to the pBR325 vector with T4 ligase, also cleaved with EcoRI. The plasmid DNA:pBR325 proportion in the binding mixture was 10:1, with an amount of DNA equivalent to 60 ng. The optimal conditions for the reaction were those specified by the manufacturers of the enzymes. Cleavage was performed at 37°C/4 h and binding at 22°C/24 h.

The binding mixture was used in the transformation of *E. coli* according to the procedure of Morrison (24), and the transformants containing the recombinant plasmids were selected by the replica plating technique (18).

Recombinant *E. coli* plasmids were transferred to *Z. mobilis* and later to *E. coli* by conjugation using the mobilizing plasmid pRK2013. The cells in conjugation were incubated for 4 hours at 30°C on a Millipore membrane (0.45 μm pore size) and maintained on agar-LB or agar-RM. The membrane was then washed with 0.8% saline and the cell suspension was centrifuged at 2,200 x g for 10 minutes. The sediment was resuspended in 0.5 mL saline and 150 mL aliquots were used for the selection of transconjugants in selective medium containing the appropriate antibiotics.

The stability of a recombinant plasmid was tested in *Z. mobilis* and *E. coli* on the basis of resistance to tetracycline. The cells were first inoculated into culture medium containing tetracycline and a colony was then cultured for several days with daily transfer to liquid medium containing no antibiotic. The number of cells resistant and sensitive to the antibiotic was determined daily.

RESULTS AND DISCUSSION

The electrophoretic pattern of plasmid DNA from *Z. mobilis* and from pBR325 treated with EcoRI is shown in FIGURE 1. Partial digestion of *Z. mobilis* plasmid DNA (FIGURE 1, column C) is important to avoid total restriction of the origins of replication of natural plasmids if sites for EcoRI are present. Cloning of DNA fragments containing the origin of replication of natural plasmids may favor the stability of recombinant plasmids in *Z. mobilis*.

The transformation of *E. coli* HB101 with the binding mixture between pBR325 and fragments of *Z. mobilis* plasmid DNA resulted in 420 tetracycline-resistant colonies, 19 of which were sensitive to chloramphenicol, indicating the insertion of *Z. mobilis* plasmid DNA fragments into the pBR325 gene for resistance to chloramphenicol. Analysis by agarose gel electrophoresis of the preparations of the 19 recombinant plasmids, denoted pZMB1 to pZMB19 (FIGURE 2), indicates the presence of plasmids of different molecular weights. EcoRI cleavage of these recombinant plasmids confirmed the cloning

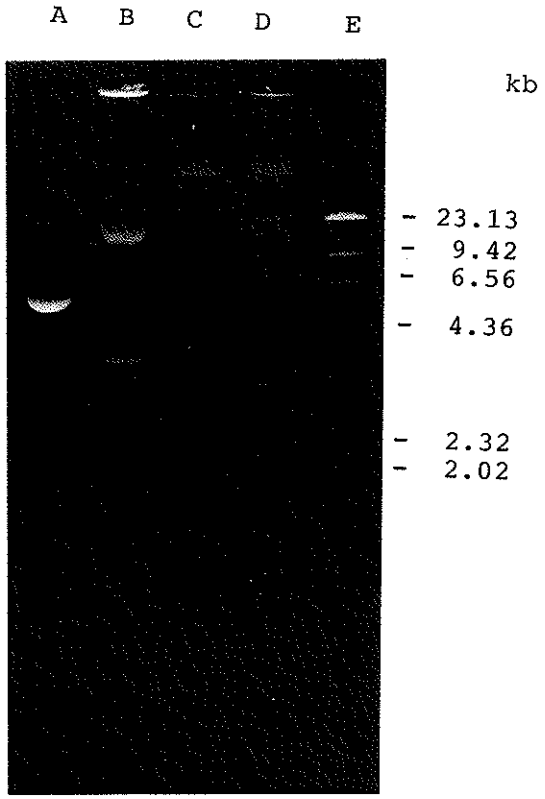


FIGURE 1 - Plasmid DNA on agarose gel: pBR325 cleaved with EcoRI (A) and uncleaved (B); total plasmid DNA of *Zymomonas mobilis* Ag11 cleaved with EcoRI (C) and uncleaved (D); DNA of the lambda phage cleaved with HindIII.

of plasmid DNA fragments of different sizes and, in some cases, the cloning of more than one plasmid fragment (FIGURE 3).

The size of the 19 plasmid DNA fragments cloned ranged from <0.5 to 8.6 kb, most of them being smaller than 3.0 kb (TABLE 2). Plasmids pZMB5, pZMB10 and pZMB11 presented two cloned DNA fragments.

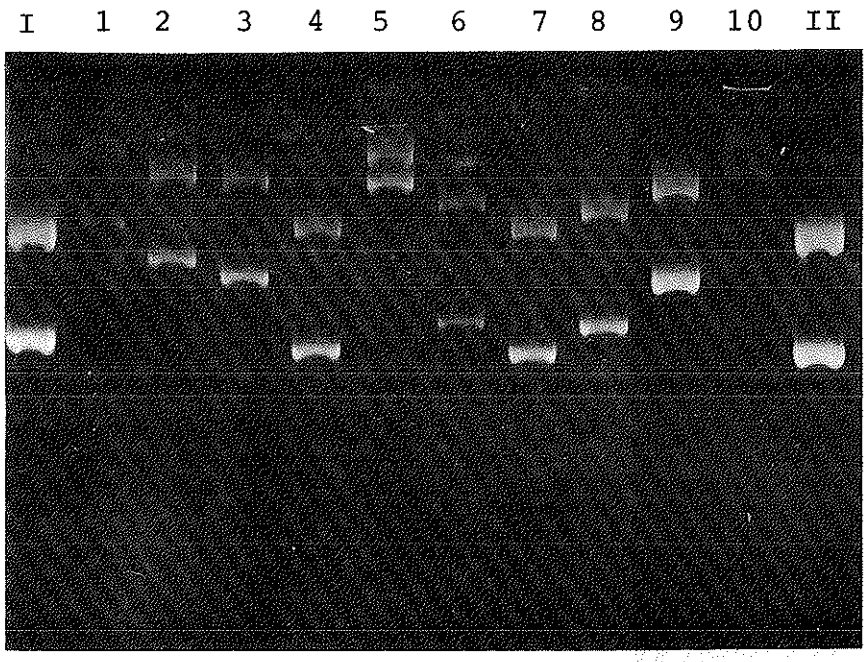
The recombinant plasmids were transferred from *E. coli* HB101 to *Z. mobilis* using pRK2013, which is considered to be an efficient agent for the mobilization of plasmid DNA in the conjugation process (12). The *Z. mobilis* transconjugants selected in tetracycline-containing RM medium were also resistant to ampicillin, indicating that pBR325 continued to be expressed. The frequency

TABLE 2 - Size of fragments of total plasmid DNA of *Zymomonas mobilis* Ag11 cloned at the EcoRI restriction site of pBR325.

Recombinant Plasmids	Size of cloned fragments (kb)
pZMB1	4.5
pZMB2	3.0
pZMB3	2.3
pZMB4	<0.9
pZMB5	1.7; 2.3
pZMB6	5.5
pZMB7	<0.9
pZMB8	1.1
pZMB9	2.4
pZMB10	1.7; 2.3
pZMB11	2.4; 2.5
pZMB12	<0.5
pZMB13	<0.5
pZMB14	<0.5
pZMB15	2.1
pZMB16	2.3
pZMB17	3.0
pZMB18	1.0
pZMB19	8.6

TABLE 3 - Frequency of transfer of the recombinant plasmids mobilized by pRK2013 of *Escherichia coli* HB101 to *Zymomonas mobilis* Ag11.

Recombinant Plasmids	Frequency of transfer per donor cell
pZMB1	3.0×10^{-7}
pZMB2	1.2×10^{-6}
pZMB3	3.3×10^{-7}
pZMB4	$<1.0 \times 10^{-8}$
pZMB5	6.9×10^{-8}
pZMB6	4.2×10^{-7}
pZMB7	1.2×10^{-6}
pZMB8	5.0×10^{-8}
pZMB9	4.8×10^{-7}
pZMB10	4.7×10^{-7}
pZMB11	5.3×10^{-7}
pZMB12	5.4×10^{-7}
pZMB13	7.0×10^{-7}
pZMB14	1.9×10^{-7}
pZMB15	6.5×10^{-7}
pZMB16	1.5×10^{-4}
pZMB17	$<1.0 \times 10^{-8}$
pZMB18	4.9×10^{-8}
pZMB19	3.2×10^{-6}



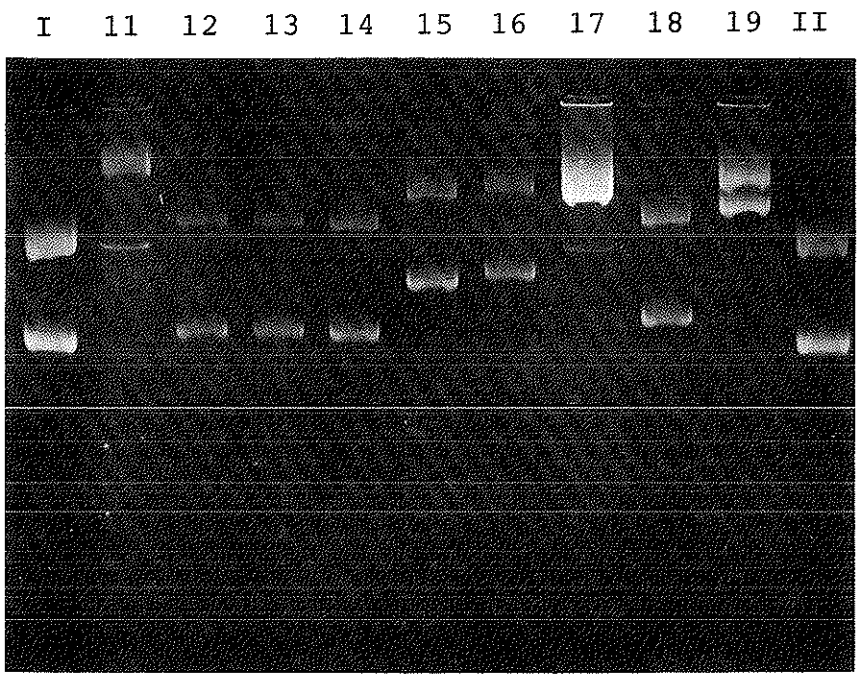


FIGURE 2 - Plasmid DNA on agarose gel: recombinant pZMB1 to pZMB19 plasmids obtained by cloning plasmid fragments of *Zymomonas mobilis* Ag11 at the EcoRI site of pBR325; pBR325 (I and II).

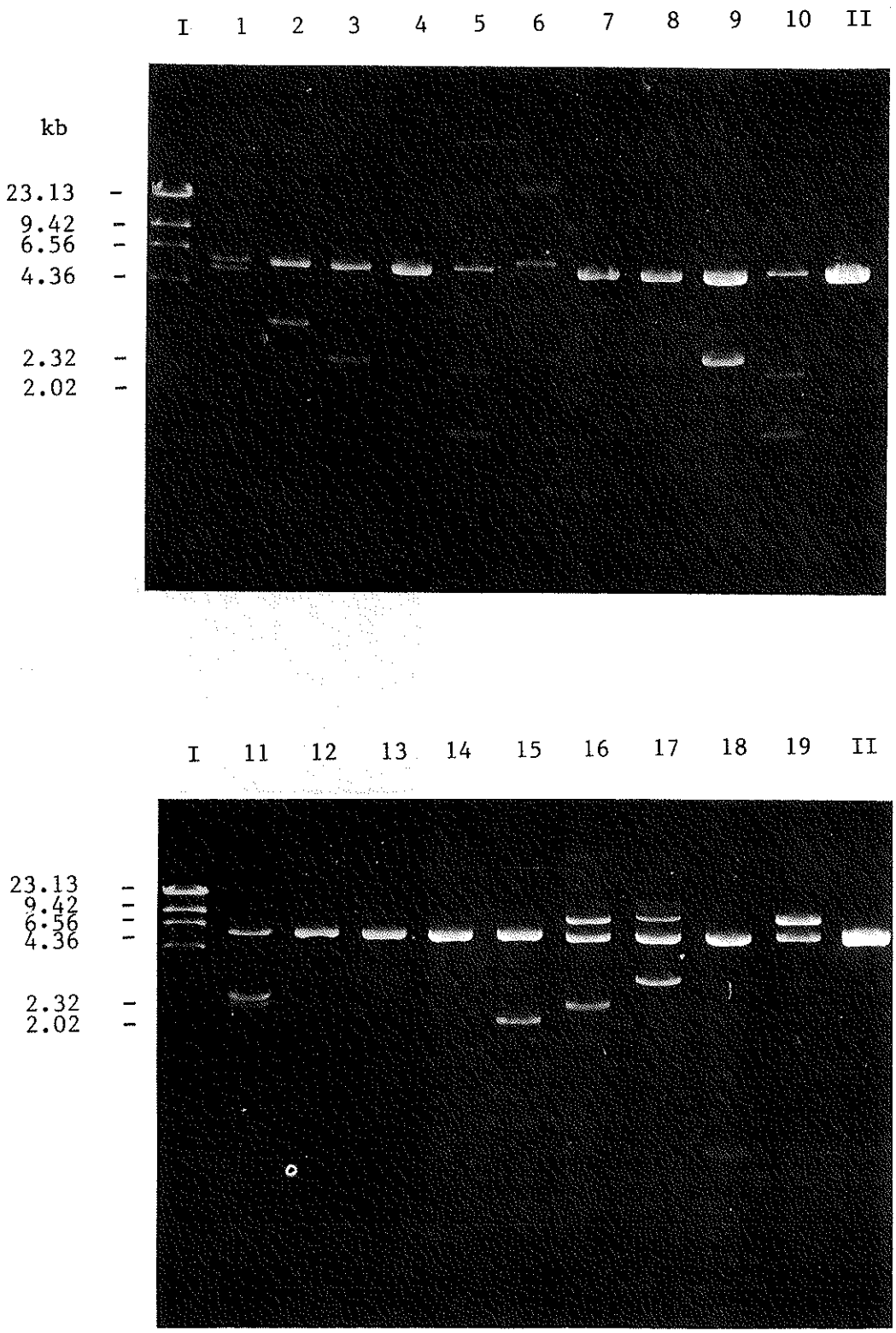


FIGURE 3- Plasmid DNA on agarose gel: recombinant plasmids pZMB1 to pZMB19 cleaved with EcoR1; lambda phage DNA cleaved with HindIII (I); pBR325 cleaved with EcoRI (II).

of mobilization of the 19 recombinant plasmids to *Z. mobilis* ranged from <1 x 10^{-8} to 1.5 x 10^{-4} transconjugants per donor cell (TABLE 3). Most of them presented a frequency of approximately 10^{-7} transconjugants per donor cell. Frequencies of about 3.5 x 10^{-7} have been obtained previously for the mobilization of pBR325 to *Z. mobilis* (1).

The electrophoretic pattern of the recombinant plasmids extracted from *Z. mobilis* did not differ from the pattern of the original *Z. mobilis* DNA plasmid. The introduction of recombinant plasmids 6.0 to 14.6 kb in size into *Z. mobilis* should have resulted in differentiation between the two patterns. The presence of recombinant plasmids in *Z. mobilis* was confirmed by the expression of tetracycline resistance by the cells in RM medium. The absence of bands corresponding to the recombinant plasmids may have been due to the small number of plasmid copies in the cells or to the recombination between DNA segments cloned in pBR325 and homologous regions in the natural plasmids of *Z. mobilis*. The stability of natural plasmids (14, 35), the efficient system of restriction and modification present in *Z. mobilis* (25), the incompatibility between plasmids of the same replication origin (10) and the presence of tetracycline in the culture medium may have favored the selection of natural *Z. mobilis* plasmids that recombined with the tetracycline gene of pBR325. This recombination in plasmids of high molecular weight is not normally visualized on the gel. Recombinant plasmids containing regions other than that of the origin of replication may also be subjected to recombination at a frequency that depends on fragment size. According to Ogale and Deobagkar (25), rearrangements between plasmid DNA and chromosomal DNA have been observed.

TABLE 4 - Stability of the recombinant plasmid pZMB12 in *E. coli* and *Zymomonas mobilis*, determined as a function of resistance to tetracycline.

Time (days)	Stability (%)	
	E. coli HB101(pZMB12)	*Z. mobilis* (pZMB12)
0	100	100
1	10	90
2	1	100
3	0.4	87
4	-	89
5	-	13
6	-	2

In the conjugation experiments of *Z. mobilis* Ag11 and *E. coli* using pRK2013 to mobilize the recombinant plasmids, only the *E. coli* transconjugant pZMB12 presented resistance to tetracycline in LB medium. This transconjugant grew in medium containing ampicillin but not in medium with chloramphenicol, indicating the presence of the recombinant plasmid in *E. coli*. The plasmid extracted from *Z. mobilis* cells was larger in size than the original pZMB12, indicating a possible insertion of the DNA fragment by recombination. This plasmid was denoted pZMBA12. EcoRI cleavage of pZMBA12 demonstrated that pBR325 maintained its original size (FIGURE 4). The transfer of pZMBA12 from

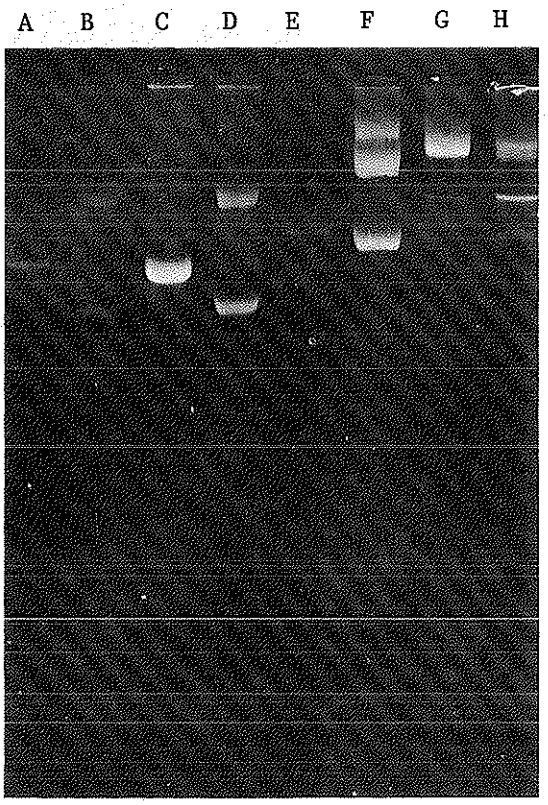


FIGURE 4 - Plasmid DNA on agarose gel: pBR325 cleaved with EcoRI (A) and uncleaved (B); recombinant pZMB12 plasmid cleaved with EcoRI (C) and uncleaved (D); recombinant pZMBA12 plasmid cleaved with EcoRI (E) and uncleaved (F); total plasmid DNA of the transconjugant Zymomonas mobilis Ag11 (pZMBA12) (G) and of Z. mobilis Ag11 (H).

E. coli DH5∝ to *E. coli* HB101 conferred tetracycline and ampicillin resistance to the cells.

The stability of the recombinant plasmid pZMB12 determined by resistance to tetracycline was lower in *E. coli* HB101 than in *Z. mobilis* (*Table* 4). Only 0.4% of the HB101 cells containing the recombinant plasmid conserved the marker for resistance to tetracycline after 3 days of growth in tetracycline-free LB medium. Under the same conditions, approximately 90% of the *Z. mobilis* cells containing the recombinant plasmid maintained the characteristic of resistance to tetracycline in RM medium. This greater stability of the marker for resistance to tetracycline in *Z. mobilis* may have originated from the recombination of plasmid DNA fragments of *Z. mobilis* in pBR325.

The construction of cloning vectors from plasmid DNA fragments of *Z. mobilis* Ag11 seems to be subjected to recombination processes and the incompatibility with natural plasmids seems to be due to the existence of homologous regions in the bacterial DNA. The existence of homologous regions and the stability of natural plasmids are characteristics that should be considered in the construction of cloning vectors in *Z. mobilis* Ag11.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support from CNPq and CAPES.

RESUMO

Estabilidade de Plasmídio Recombinante em *Zymomonas mobilis* Ag11

Foram obtidos 19 plasmídios recombinantes contendo fragmentos de DNA plasmidial de *Zymomonas mobilis* Ag11, clonados no sítio de EcoRI do pBR325. Os fragmentos clonados variaram de <0,5 a 8,6 kb. O plasmídio recombinante pZMB12 teve seu peso aumentado, por processos de recombinação, durante sua permanência em *Z. mobilis* Ag11. Este plasmídio denominado pZMBA12, ao ser clivado com EcoRI mostrou que o pBR325 permaneceu com seu tamanho original. O plasmídio recombinante pZMB12 apresentou maior estabilidade em *Z. mobilis* do que em *E. coli*.

Palavras-chave: *Zymomonas mobilis*, plasmídio recombinante

REFERENCES

1. Afendras, A.S.; Drainas, C. Expression and stability of a recombinant plasmid in *Zymomonas mobilis* and *Escherichia coli*. *J. Gen. Microbiol.*, 133: 127-134, 1987.
2. Bethesda Research Laboratories. BRL PUC host: *Escherichia coli* DH5∝ competent cells. *Focus*, 8:9, 1986.
3. Bimboim, H.C.; Doly, J.A. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acid. Res.*, 7: 1513-1523, 1979.
4. Bolivar, F. Molecular cloning vehicles derived from ColE1. type plasmid pMB1. *Life Science*, 25: 807-818, 1979.
5. Boyer, H.W.; Rouland-Dussoix, D. A complementation analysis of restriction and modification of the DNA in *Escherichia coli*. *J. Mol. Biol.*, 41: 459-472, 1969.
6. Byun, M.O,K.; Kaper, J.B.; Ingram, L.O. Construction of a new vector for the expression of foreign genes in *Zymomonas mobilis*. *J. Indust. Microbiol.*, 1:9-15, 1986.
7. Carey, V.C.; Walia, S.K.; Ingram, L.O. Expression of lactose transposon (Tn951) in *Zymomonas mobilis*. *Appl. Environ. Microbiol.*, 46:1163-1168, 1983.
8. Cho, D.; Rogers, P.L.; DeLaney, S.F. Construction of a shuttle vector for *Zymomonas mobilis*. *Appl. Microbiol. Biotechnol.*, 32:50-53, 1989.
9. Conway, T.; Byun, M.O.K.; Ingram, L.O. Expression vector for *Zymomonas mobilis*. *Appl. Environ. Microbiol.*, 53: 235-241, 1987.
10. Couturier, M.; Bex, F.; Bergquist, P.L.; Maas, W.K. Identification and classification of bacterial plasmid. *Microbiol. Rev.*, 52:375-395, 1988.
11. Dally, E.L.; Stokes, H.W.; Eveleigh, D.E. A genetic comparison of strains of *Zymomonas mobilis* by a analysis of plasmid DNA. *Biotechnol. Lett.*, 4:91-96, 1982.
12. Ditta, G.; Satnfield, S.; Corbin, D.; Helinski, D.R. Broad host range DNA cloning system from gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. USA*, 77:7347-7351, 1980.
13. Drainas, C.; Slater, A.A.; Coggins, L.; Montagne. P.; Corta, R.G.; Ledingham, W.M.; Kinghorn, J.R. Electron microscopic analysis of *Zymomonas mobilis* strain ATCC 10988 plasmid DNA. *Biotechnol.Lett.*, 5:405-408, 1983.
14. Drainas, C.; Typas, M.A.; Kinghorn, J.R. A derivative of *Zymomonas mobilis* ATCC strain 10988 with impaired ethanol production. *Biotechnol. Lett.*, 6:37-42, 1984.
15. Eveleigh, D.H.; Stokes, H.W.; Dally, E.L. Recombinant DNA approaches for enhancing the ethanol productivity of *Zymomonas mobilis* In: WISE, D.L. (ed.) *Organic Chemicals from Biomass*. Menlo Park, CA, Benjamin Cummings. 1983. p.69-91.
16. Figurski, D.H.; Helinski, D.R. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA.*, 75:1648-1652, 1979.
17. Hanahan, D. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, 166:577-580, 1983.
18. Lederberg, J.; Lederberg, E.M. Replica plating and indirect selection of bacterial mutants. *J. Bacteriol.*, 63:399-406, 1952.

19. Lima, O.G.; Larios, C.; Azcarate, E. Aislamento y estudio de nuevas cepas de *Pseudomonas lindneri* Kluyver et Hoppenbrouwers (*Termobacterium mobile* Lindner), en aguamieles de la meseta Central Mexicana. *Ciência,* 11:273-277, 1951.
20. Maniatis, T.; Fritsch, E.F.; Sambrook, J. *Molecular Cloning:* A laboratory manual. Cold Spring Harbor, NY. Cold Spring Harbor Laboratory. 1982. 545p.
21. Miller, J.H. *Experiments in Molecular Genetics*. Cold Spring Harbor, NY. Cold Spring Harbor Laboratory. 1972. 430 p.
22. Misawa, N.; Okamoto, T. Nakamura, K.; Kitamura, K.; Yanase, H.; Tonomura, K. Construction of a new shuttle vector for *Zymomonas mobilis. Agric. Biol. Chem.*, 50:3201-3203, 1986.
23. Misawa, N.; Okamoto, T.; Naramura, K. Expression of a cellulase gene in *Zymomonas mobilis. J. Bacteriol.*, 7:167-178, 1988.
24. Morrison, D.A. Transformation in *Escherichia coli*: cryogenic preservation of competent cells. *J. Bacteriol.*, 132: 349-351, 1977.
25. Ogale, S.S.; Deobagkar, D.N. A high molecular weight plasmid of *Zymomonas mobilis* harbours genes for $HgCl_2$ resistance. *Biotechnol. Lett.*, 10:43-48, 1988.
26. Rogers, P.L.; Lee, K.J.; Tribe, D.E. High productivity ethanol fermentation with *Zymomonas mobilis. Proc. Biochem.*, 15:7-11, 1980.
27. Rogers, P.L.; Skotnicki, M.L.; Lee, K.J.; Lee, J.H. Recent developments in the *Zymomonas* process for ethanol production. *CRC. Crit. Rev. Biotechnol.*, 1:273-285, 1984.
28. Schaffer, H.E.; Sederoff, R.R. Improved estimation of DNA fragment length from agarose gels. *Anal. Biochem.*, 115:113-122, 1981.
29. Scordaki, A.; Drainas, C. Analysis of natural plasmids of *Zymomonas mobilis* ATCC 10988. *J. Gen. Microbiol.*, 133: 2547-2556, 1987.
30. Skotnicki, M.L.; Tribe, D.E.; Rogers, P.L. R-plasmid transfer in *Zymomonas mobilis. Appl. Environ. Microbiol.*, 40:7-12, 1980.
31. Skotnicki, M.L.; Goodman, A.E.; Warr, R.G.; Rogers, P.L. Isolation and characterization of *Zymomonas mobilis* plasmids. *Microbios,* 40:53-61, 1984.
32. Strzelecki, A.T.; Googman, A.E.; Rogers, P.L. Stability of the *lac* operon in *Zymomonas mobilis* in batch and continuous culture. *J. Bacteriol.*, 3:197-205, 1986.
33. Swings, J.; DeLey, J. The biology of *Zymomonas mobilis Bacteriol. Rev.*, 41:1-46, 1977.
34. Tonomura, K.; Okamoto, T.; Yasui, M.; Yanase, H. Shuttle vectors for *Zymomonas mobilis. Agric. Biol. Chem.*, 50:805-808, 1986.
35. Yablonsky, M.D.; Goodman, A.E.; Stevnsborg, N.; Lima, O.G.; Morais, J.O.F.; Lawford, H.G.; Rogers, P L.; Eveleigh, D.E. *Zymomonas mobilis* CP4: a classification of strains via plasmid profiles. *J. Biotechnol.*, 9:71-80, 1988.

Rev. Microbiol., São Paulo, **26**(3): 211-218, 1995

GROWTH AND COMPETITION BETWEEN TWO STRAINS OF *BRADYRHIZOBIUM JAPONICUM* IN BROTH AND IN A PEAT-BASED INOCULANT: DINITROGEN FIXATION EFFICIENCY AND COMPETITION FOR NODULATION SITES.*

Claudio Luis Crescente Frankenberg[1]
João Ruy Jardim Freire[2**]
Robert Wayne Steven Philip Thomas[3]

ABSTRACT

Bradyrhizobium japonicum strains *SEMIA* 587 and *SEMIA* 5019 were evaluated for growth and competition in liquid broth cultures and in sterilized peat. Nodulation ability, strain nodule occupancy rates and dinitrogen fixation were investigated in Leonard jar experiments using soybean plants as host. *B. japonicum* strains *SEMIA* 587 and 5019 were grown together in a bench-top fermentor or separately in shake-flask cultures, using glycerol as primary carbon source. Maximum growth rate and generation time occurred at 28°C and 250 rpm. Maximum productivity was at 28°C and 300 rpm. Shake-flask cultures inoculated with equal numbers of both strains contained 69% of strain 587 and 31% of strain 5019 after 120 hours (double-strain broth), due to the lower growth rate and productivity of strain 5019. When double-strain broth was added to sterilized peat only 10.2% of strain 5019 was recovered after 180 days of incubation, but when sterilized peat was inoculated with a 1:1 (v/v) mixture of the strains, 37% of strain 5019 was recovered after the same time period. Total cell numbers increased during 30 days of incubation at 24°C-25°C and then declined. Mixed inoculi containing both strains generally showed higher nodule occupancy rates for strain 587.

INTRODUCTION

Peat is the main carrier-material used for legume inoculants produced in Brazil, only a small part being sterilized by gamma irradiation or autoclaving. Brazilian Federal regulations stipulate that manufacturers must use strains recommended by research laboratories only. This regulation also specifies that the carrier used to dilute the culture should be inert and sterile.

According to Bonomi *et al.* (5), many technical problems still affect the quality of the final product, e.g. long fermentation times, inadequately designed fermentors, difficulty in maintaining axenic cultures, lack of information on the growth kinetics of the strains used, little information on peat quality and sterilization, scarcity of data regarding the growth and survival of the strains in peat, and inefficient and time-consuming methods for quality control of the finished product.

* This work was conducted by the first author as part of his M.Sc. course on Agricultural and Environmental Microbiology, Faculty of Agronomy, UFRGS, Brazil.

1 Catholic University of Porto Alegre, Avenida Ipiranga, Porto Alegre, Rio Grande do Sul, Brazil.

2,3 Department of Soil Sciences, Faculty of Agricultural Siences, University of Rio Grande do Sul, - UFRGS, Avenida Bento Gonçalves, Porto Alegre, Rio Grande do Sul, Brazil.

** To whom Correspondence should be sent: FAX (+55) 51 336 1211

From 1979 til 1992, strains SEMIA 587 and SEMIA 5019 (+ 29 W) were the recommended strains for soybean inoculation. In 1992, two new strains were also recommended at the meeting of the research institutions (RELARE). The four selected strains could be used either separately or in pairs. The procedure for multi-strain inoculants recommends that strains should be grown separately and the broths mixed prior to impregnation of the carrier. However, in practice, the manufacturers grow each strain separately only as starter cultures, growing both strains simultaneously in the same fermentor at production level. Laboratory tests at IPAGRO have provided evidence that while there is a predominance of strain 587, the other strain, 5019, maintains satisfactory cell numbers in both the fermentation broth and the peat carrier.

The aims of the present investigation were to determine the influence of temperature and agitation on the growth of *B. japonicum* strains in liquid media in a fermentor; investigate the competition between the two strains grown together; assess the symbiotic capacity of the strains grown in fermentors, both axenically and in pairs; evaluate competition for nodule sites and determine cell multiplication and survival rates when mixed inoculi are added to sterilized peat.

Bradyrhizobium japonicum can enter into symbiosis with soybean; it is a slow-growing bacterium that, when cultured in an industrial scale, presents some problems mainly related to the magnitude of industrial processing of cells in broth culture. Some difficulties are also encountered regarding *B. japonicum* multiplication and survival if non-sterile peat is used as carrier material. Lopreto *et al.* (21) and Bonomi *et al.* (6) studied the effect of aeration on broth cultures of *B. japonicum*, while Bonomi (5) studied the control of pH. Many other authors have investigated the effects of aeration, agitation, temperature, pH and medium composition on the multiplication of *B. japonicum* in liquid media (2, 3, 8, 9, 13, 14, 20, 22, 27, 34).

According to Brunello and Concone (7), agitation and aeration are included in a fermentation process to facilitate gas dispersion, accelerate mass-transport, suspend solid particles (including microorganisms), disperse unmiscible liquids and facilitate heat transfer. Thiemann (31) remarked that agitation affects aeration by increasing the liquid/gas interface. This delays the passage of gas through the medium and thus allows better gas transfer, avoiding the coalescence of gas bubbles and increasing liquid turbulence.

Ertola *et al.* (12) investigated the relationship between agitation, medium composition and oxygen supply regarding the growth of *Rhizobium meliloti*. They observed that increased oxygen supply resulted in a reduction of productivity in terms of cell numbers, which they attributed to greater medium viscosity due to exopolysaccharide production. Ballati and Mazza (4) also reported that the growth of *R. meliloti* in liquid media differed depending on agitation rate and medium composition.

Lopreto *et al.* (21) described the reduced growth of *B. japonicum* under low agitation rates (100 rpm) due to low oxygen transfer rates. Mazza *et al.* (23) found that the best agitation rate was 200 rpm.

Competition for nodule sites has been studied by various authors (9, 15, 16, 32, 36), but little work has been done concerning competition between strains grown jointly in liquid culture medium or in peat.

MATERIALS AND METHODS

The experiments were carried out in the laboratories of the Science and Technology State Foundation (*CIENTEC*) and the Microbiology Laboratory of the Faculty of Agriculture, Federal University of Rio Grande do Sul (*UFRGS*).

The *Bradyrhizobium japonicum* strains used were *SEMIA* 587 (isolate 6/1967, *IPAGRO*) and *SEMIA* 5019 (isolate 29W, CNBS, Rio de Janeiro, Brazil), both supplied by the Rhizobial Culture Collection at *IPAGRO*. These strains present high dinitrogen-fixing efficiency, antibiotic resistance and competitiveness against other strains in greenhouse and field trails, strain 587 being especially competitive (15). They belong to different serogroups and can be easily distinguished by the serological agglutination method (36).

Cell population kinetics were examined in a bench fermentor with a 5 liters capacity, with a working volume of 2.5 liters. The influence of agitation on cell multiplication rate was studied on strain 587 at a constant temperature of 28 °C ± 1 °C and agitation rates of 100, 150, 200, 230, 250, 300 and 500 rpm, with an aeration rate of 0.5 v/v using

air. The culture medium (33) was adjusted to pH 7.2, sterilized and inoculated with 25mL of a starter culture which had been stored frozen in tubes. For each fermentation, one tube was used for inoculation while a duplicate tube was used to assess viable cell numbers. Four replicate fermentations were made for each experimental condition. Samples were taken at 10 sampling times for up to 120 hours of growth. Viable cell numbers were evaluated by the dilution plating method and the results expressed as colony forming units per mL (cfu mL^{-1}).

For each agitation rate, population growth curves were constructed from the data, and generation times were calculated for the exponential part of the curve (7) using the equations recommended by Murdock and Hatcher (24).

Growth rates during the exponential phase and maximum growth specific velocity were obtained using the program proposed by Ledluy and Zajic (17). The experimental error which affected these values was computed according to Borzani (9). Maximum productivity was calculated using Levean and Bouix's equation (18).

The influence of temperature was tested at 22, 26, 28, 30, 34 and 38 °C for individual strains, with an aeration rate of 0.5 v/v/minute and agitation rate of 230 rpm. Four replicates were made for each temperature. Values were computed using the same equations described above.

Competition between strains in liquid media was determined at 28°C ± 1 °C and 230 rpm, the inoculum consisting of equal numbers of viable cells of both strains. Cell population growth was studied for strains grown singly and for the two strains mixed at a 1:1 proportion. Samples were taken after 0, 24, 48, 72, 94 and 120 hours of culture and viable cell numbers determined using the dilution plating method. Glycerol consumption was measured by a standard method (1). For the various experiments, exponential growth rate, specific velocity of maximum growth and maximum productivity were determined using the same equations, but substrate conversion (Y) was additionally determined for this experiment according to the equation of Bonomi *et al.* (7).

Colonies obtained with the dilution plating method were transferred to agar slants and subsequently identified by serological agglutination as described by Vincent (35), using antisera supplied by the *IPAGRO* Institute. The numbers of colonies identified were 1066 at 24 hours and 1680 at 120 hours.

Dinitrogen-fixation efficiency and competitiveness of strains 587 and 5019 for nodule sites were tested in Leonard jars using sand and Specht's *et al.* nutrient solution (30). One treatment condition (TF1) was established using double-strain broth that contained 69% of strain 587 and 31% of strain 5019 after 120 hours of fermentation, which had started with equal quantities of the two strains. Other treatment conditions were obtained by mixing strains grown singly in shake-flask cultures, cell numbers being adequately adjusted by turbidity since the strains used did not produce significant quantities of exopolysaccharide. Calibration curves were constructed for each strain plotting turbidity against cell numbers. The treatment conditions tested were: TP1: 69% of strain 587 plus 31% of strain 5019; TP2: 50% of strain 587 plus 50% of strain 5019; and TP3: 31% of strain 587 plus 69% of strain 5019. Plant and nodule dry matter were determined by drying at 65 °C for 72 hours. Total nitrogen was estimated by the semi-micro Kjeldahl method. Nodule occupancy of individual strains was defined by serological agglutination.

Competition and survival of strains 587 and 5019 in sterilized peat as carrier material was evaluated according to standard methods (27, 29). Peat was dispensed inside polypropylene bags (wall thickness, 15 micrometers), autoclaved for 25 minutes on three consecutive days, inoculated with broth cultures (TF1, TP1, TP2, TP3), adjusted to give a final moisture content of 40 to 50% available water, and then stored at 24-25 °C. Viable cell numbers were evaluated by the plate-dilution method, 8 samples being taken from day 0 to day 120. The identity of colonies was verified by agglutination as previously described (35).

RESULTS AND DISCUSSION

Effect of Temperature on the Growth of Strain *SEMIA* 587 in Liquid Media

TABLE 1 shows that the highest growth rates and cell productivity values for strain 587 were obtained within a temperature range of 28 to 30 °C, the highest growth rate being at 30 °C and maximum

TABLE 1. Exponential growth of *Bradyrhizobium japonicum* strain *SEMIA* 587 in Mazza's medium at 230 rpm in a 2.5 litre working capacity fermentor.

Temperature °C	Exponential Phase Start (H)	Exponential Phase Duration (H)	Growth Rate ($x10^8$ CFU mL^{-1}) 10^{11} CFU mL^{-1})	Productivity (cells $H^{-1}x$	Generation Time (H^{-1})
22	22.0	19.3b*	1.5c*	0.7b*	8.8abc*
26	10.6	32.3a	2.3bc	1.33b	10.8a
28	8.8	27.6ab	4.8ab	4.75a	7.5c
30	9.6	29.6a	6.0a	4.64a	7.7bc
34	9.6	26.3ab	2.7bc	2.41ab	9.0ab
38	20.3	20.3b	1.5c	1.04b	10.3a

*Values followed by the same letter show no significant difference (P=0.05, Duncan's Test)

productivity at 28°C. Mean generation time at these temperatures was 7.6 hours. On the whole, the range of generation times is in good agreement with that presented by Lopreto *et al.* (20, 22), who obtained values between 9.2 and 12.3 hours for 8 strains of *B. japonicum* grown with glycerol as carbon source. Broth pH varied with temperature, being 7.16 at 22°C, 7.33 at 28°C and 7.46 at 34°C. Exponential growth started 8.8 hours after inoculation at 28°C, but at 22°C the lag/log transition occurred only after 22 hours. The average duration of exponential growth was 27.8 hours.

Effect of Agitation Rate on the Growth of strain *SEMIA* 587

TABLE 2 shows that, at 28 °C, strain 587 had a maximum growth rate and shortest generation time at 250 rpm but maximum productivity at 300 rpm, when the duration of the exponential phase was shortest. These results are in agreement with the data obtained by other workers (5, 21, 22).

Depending on agitation rate, exponential growth started between 6.9 and 20.5 hours after inoculation and lasted for 14.6 to 40.2 hours. The specific growth rate constant varied from 0.044 at 100 rpm to 0.098 at 250 rpm.

No statistical differences were found between the different growth rate values except for growth at 250 rpm, which was 279% higher than the next highest values detected at 230 and 300rpm.

Starting with an inoculum of 8.0×10^8 cfu mL^{-1}, cell numbers reached 4.0×10^{10} cfu mL^{-1} after 72 hours of agitation at 230 rpm, the pH rising from 7.1 to 7.57. These numbers are low compared to those described by Balatti (3) and Mazza et al (23), who reported reaching 10^{10} cfu mL^{-1} after 40 hours of growth in column-type fermentors.

TABLE 2. Exponential growth of *Bradyrhizobium japonicum* strain *SEMIA* 587 in Mazza's medium at 28°C in a 2.5 litre working capacity fermentor.

Agitation Rate (RPM)	Exponential Phase Start (H)	Exponential Phase Duration (H)	Growth Rate ($x10^8$ CFU mL^{-1}) 10^{11} CFU mL^{-1})	Productivity (cells $H^{-1}x$	Generation Time (H^{-1})
100	20.5	40.2	2.4b*	1.5c*	14.8a*
150	12.0	33.0	1.9b	1.6c	14.7a
200	7.6	30.0	3.2b	3.1abc	9.3b
230	8.8	27.6	4.8b	4.7ab	7.5b
250	9.3	33.0	13.4a	4.6ab	6.5b
270	6.9	20.4	2.9b	4.5ab	7.2b
300	15.6	14.6	4.8b	4.9a	7.0b
500	7.2	30.0	2.6b	2.5bc	9.7ab

*Values followed by the same letter show no significant difference (P=0.05, Duncan's Test)

Competition Between Strains *SEMIA* 587 and *SEMIA 5019* in Liquid Media

Results on the simultaneous growth of the two *B. japonicum* strains are shown in TABLE 3. It can be observed that strain 587 outcompeted strain 5019 tough, interestingly, the duration of exponential growth was longer for the latter. Evaluating the mixture as a whole, it can be seen that the values recorded fall within the range of intermediate individual values for the two strains. It should be pointed out, however, that the growth conditions used were those previously defined as optimal for strain 587. Different results might thus have been obtained under growth conditions more favorable to strain 5019. The generation times obtained are in agreement with those reported by other authors (20, 22, 23).

After 24 of hours growth in liquid medium, plating and serological identification showed that 53% of the plated colonies belonged to strain 587, while 46% belonged to strain 5019. The proportion of strain 583 had risen to 69% after 120 hours. Since the initial inoculum contained equal numbers of each strain, this result indicates that *B. japonicum* strain 587 outperformed strain 5019 under the culture conditions used, as could indeed be expected considering the longer generation time of strain 5019.

This highlights the difficulties associated with the simultaneous growth of distinct strains in the same fermentation vessel, a practice which is common among inoculant manufactures in Brazil.

Strain 587 consumed 90% of the carbon source (glycerol) after 120 hours of culture, while the mixture of the two strains consumed 87% and strain 5019 only 57% for the same time period. Biomass production (0.566 grams of cells per gram of glycerol) was higher than would have been predicted considering glycerol as the only carbon source. This may have been due to the cultures utilizing yeast extract present in the medium as an additional source of carbon (5).

Survival and Competition Between Strains *SEMIA* 587 and *SEMIA* 5019 in Sterilized Peat Carrier

When double-strain broth TF1 (containing 69% of strain 587 and 31% of strain 5019) was added to sterilized peat carrier, no significant differences were found between the numbers of strain 587 colonies recovered on plating and those recovered when the strain was inoculated into peat carrier as pure culture. The use of mixture TP2 revealed differences in survival for strain 5019. The results show a general increase in cell numbers during the first 30 days of growth, followed by an abrupt decrease during the next 60 days and gradual reduction over the last 90 days. Cell mortality rates agree with those described by other workers (9, 13, 14, 33).

Evaluation of Dinitrogen-Fixation and Nodule Occupancy

Many researchers have studied competition for nodule occupancy between rhizobial strains. Trinick (32) remarked that it is influenced by both host and symbiont characteristics (competition, physiology, growth rate) as well as by environmental factors such as temperature, water activity and soil nutrients.

Several workers (11, 26, 28) have observed that very competitive strains can dominate nodulation even when present in the inoculant at lower numbers than those of less competitive strains.

TABLE 3. Exponential growth of *Bradyrhizobium japonicum* in Mazza's medium at 28°C in a 2.5 litre working capacity fermentor at 230 rpm.

Strain	Duration of Exponential Growth (H)	Growth Rate ($x10^8$ CFU mL^{-1})	Productivity (Cells H^{-1} x 10^{11} CFU mL^{-1})	Generation Time (H^{-1})
587	27.6	4.1	4.0	8.2
5019	63.3	1.8	1.3	15.0
587+5019	49.0	3.7	2.7	10.1

TABLE 4. Percentage occurrence of *Bradyrhizobium japonicum* strains *SEMIA* 587 and *SEMIA* 5019 in sterilised peat inoculants impregnated with fermentor broth (TF1, 69% 587 + 31% 5019) or with a mixture of broths grown seperately (TP2, 50% of each strain). Colonies were typed by a serological agglutination method, using samples taken from 4 inoculant packages.

	Broth TF1			Broth TP2		
Incubation Period (Days)	Number of Colonies Typed	Serogroup (%) 587	5019	Number of Colonies Typed	Serogroup (%) 587	5019
0	974	70.03	29.97	880	50.23	49.77
15	768	71.17	28.83	840	52.74	47.26
30	716	73.34	26.66	818	57.77	42.23
60	1044	78.40	21.60	1148	60.89	39.11
90	1168	85.03	14.97	654	63.19	36.81
120	922	89.09	10.91	778	63.96	36.04
150	1002	89.25	10.75	1310	64.35	35.65
180	810	89.76	10.24	1004	65.23	34.77

TABLE 5. Nodule typification by serological agglutination, number and weight of nodules, dry matter content and total Nitrogen content. Results on soybean growth in Leonard jars inoculated with pure cultures or mixtures of *Bradyrhizobium japonicum* strains *SEMIA* 587 and *SEMIA* 5019.

Treatment	Number of Nodules/Jar (mg)	Weight of Nodules/Jar (g/jar)	Plant Dry Matter	Total Nitrogen (mg/Jar)	Nodule Occupancy* (%) 587	5019
SEMIA 587	88c**	296ab	4.12b	124b	100.0	0.0
SEMIA 5019	130ab	311a	3.86b	113b	0.0	100.0
TF1***	151a	282ab	4.54b	142b	97.7	2.3
TP1	92c	201bc	4.28b	126b	81.8	18.2
TP2	106bc	254ab	4.17b	119b	59.1	40.9
TP3	95c	120c	2.64bc	64bc	42.2	52.8
Control	0	0	1.27c	16c	——	——
Control+N	0	0	6.69a	251a	——	——

* A low percentage of nodules that showed no reaction or double occupancy were not considered in the calculations. The number of nodules tested by treatment were: *SEMIA* 587=124, *SEMIA* 5019=51, TF1=103, TP1=140, TP2=312, TP3=1656.

**Means of five replicates. Values followed by the same letter were not significantly different according Duncan's test (P=0.05)

*** Treatment	% Strain 587	+ 5019	Production Method
TF1	69.07	30.95	Joint growth in fermentor
TP1	70.00	30.00	Strains grown singly in flasks
TP2	50.00	50.00	Strains grown singly in flasks
TP3	30.00	70.00	Strains grown singly in flasks

Another factor to consider is adaptation to a given soil environment. Freire *et al.* (15) studied spread during a ten years period and showed that less competitive strains can increase their nodule occupation over the years.

In the present study, results from greenhouse tests using soybean grown in Leonard jars showed that total nitrogen content presented a high correlation (r = 0.99) with plant-top dry weight but a low correlation (r = 0.72) with nodule weight. These results differ from data reported by some other authors (10, 19, 34, 26).

The serological identification of nodule-infecting symbionts showed that 42% of the soybean nodules from the Leonard jar experiments were produced by *B. japonicum* strain 587 when 31% (plants inoculated with TP3 mixtures) of this strain was present in the original peat inoculum. However, occupancy rates rose to 98% when strain 587 accounted for 69% of bacterial cells in the original inoculum (plants inoculated with TP1 mixtures.

The predominant nodule occupancy of one strain in relation to its partner in an inoculant mixture, or to native soil rhizobia, could explain the plant net nitrogen content. Results from trials with inoculant mixtures containing equal proportions of each strain tested (plants inoculated with TP2 mixtures) showed that strain 587 predominated over strain 5019, probably due to the better adaptability of the former. There was no statistically significant difference between the various treatments, but TP3 mixtures (containing 31% of strain 587 and 69% of strain 5019) presented consistently lower levels of nodulation.

CONCLUSIONS

These results illustrate the importance of studies designed to evaluate compatibility between different rhizobial strains when they are destined for inclusion in multi-strain inoculants. Recommendations for the production of these inoculants must be preceded by careful laboratory studies and also by studies related to large-scale production, where growth conditions may be different. Other important points to consider are the need for better methods of identification of individual strains and more laboratory, greenhouse and field experiments under different environmental conditions. These investigations would facilitate to a large extent the process of recommending single-strain inoculants.

RESUMO

Crescimento e competição entre duas estirpes de *Bradyrhizobium japonicum* em caldo e em um inoculante turfoso: eficiência na fixação do dinitrogênio e competição por sitios de nodulação.

Este trabalho relata uma pesquisa sobre o crescimento e competição em meio líquido, em inoculante em veículo turfoso e por sitios de nodulação entre duas estirpes de *Bradyrhizobium japonicum* recomendadas para produção de inoculante para soja. Foi avaliada também a eficiência na fixação do nitrogênio. As estirpes SEMIA 587 e SEMIA 5019 foram cultivadas em mistura ou isoladamente em um fermentador de bancada e em frascos em agitador. Taxa de crescimento máximo e menor tempo de geração ocorreu a 28°C e 250 rpm. Caldo semeado com igual concentração de ambas as estirpes após 120 horas apresentava concentração de 69% da 587 e 31% 5019 devido a menor taxa de crescimento e maior tempo de geração deste estirpe. Em um inoculante turfoso estéril a mistura das estirpes na concentração citada acima é com concentração 1:1, após 180 dias de incubação apresentava somente 10,2% e 34.7% da estirpe 5019 respectivamente. A concentração celular aumentava por 30 dias e declinava após drasticamente. Em experimento em vasos Leonard a inoculação da mistura das estirpes resultou maior ocupação dos nódulos pela estirpe 587. Esta estirpe também apresentou fixação do nitrogênio mais alta.

Palavras chave: *Bradyrhizobium japonicum*, soja, fixação do nitrogênio.

REFERENCES

1. Anon. - *Determinação de Glicerol por Oxidação por Periodate*. Internal document DQEQ/AB:01.01, Instituto de Pesquisas Tecnológicas, São Paulo, São Paulo, Brazil, 1982, pp.4.
2. Balatti, A.P. - *Produccion de inoculantes*. La Plata, Centro de investigaciones y desarrollo de Fermentaciones Industriales, Faculdade de Ciencias exactas, La Plata, Argentina, 1976, pp. 32.
3. Balatti, A.P., - Culturing *Rhizobium* in large-scale fermentors. *BNF Technology for Tropical agriculture*, 127-132, 1981.
4. Balatti, A.P.; Mazza, L.A., - Obtencion de inoculantes para soja. *Rev. Arg. Microbiol.*, 11: 83-88, 1979.
5. Bonomi, A,; Barral, M.F.; Koshimizu, L.H.; Mantelatto, P.E.; Ohba, M.S.; Afonso, M. - Desenvolvomento technológico para a produção industrial de *Rhizobium japonicum*. *Fertilizantes*, 8: 3-7, 1986.
6. Bonomi, A.; Carvalho, R.N; Oliveira, M.S.; Pradella, J.G.C.; Severo, R.; Urenha, L.C. - Influéncia da acração no crescimento de *Rhizobium japonicum* esterpe 587 em fermentador. *Primero Encontro Brasil, Argentina e Uruguai sobre inoculantes.*, Porto Alegre, Rio Grande do Sul, Brazil, 1987.
7. Brunello, G.; Concone, B.R.V. - Agitação e aeração em

fermentadores. *In*: Borzani *et al* - *Engenharia Bioquímica*, Edgar Blucher, São Paulo, Brazil, 3: 137-167. 1975,

8. Burton, J.C. -*Rhizobium* culture and use. In: Peppler, H.J., ed.,- *Microbial Technology*, Van Norstrand Reinhold, New York, U.S.A., 1-33, 1967.
9. Date, R.A. - Legume inoculant production. *Proceedings of the Indian National Science Academy, Section B*, Bangalore City, India, 40: 667-686, 1976.
10. Dionisio, J.A. - *Eficiência e Competição de Estirpes de Rhizobium japonicum na Cultura de Soja.* Masters Dissertation, Faculdade de Agronomia, UFRGS, Porto Alegre, Rio Grande do Sul, Brazil, 1985, pp. 91.
11. Ellis, W.R.; Ham, G.E.; Schmidt, E.L. - Persistence and recovery of *Rhizobium japonicum* inoculum in a field. *Soil Agron. J.*, 76: 573-576, 1984.
12. Ertola, R.J.; Balatti, A.P.; Cuevas, C.M. and Daguerre, R. - Effect of composition of medium and oxygen supply rates on growth of *Rhizobium meliloti*. *Soil Science*, 108: 373-330, 1969.
13. Freire, J.R.J.; Jones, S.H. - Influência da temperatura de armazenagem e da perda de humididade na longevidade dos inoculantes de leguminosas. *Revista Faculdade de Agronomia e Veterinária*, UFRGS, Porto Alegre, Riogrande do Sul, Brazil, 6: 85-93, 1963.
14. Freire, J.R.J.; Jones, S.H. - Longevidade dos inoculantes de leguminosas. *Revista Faculdade de Agronomia e Veterinária*, Universidade Federal do Rio Grande do Sul, Porto Alegre, Riogrande do Sul, Brazil, 1: 29-34, 1964.
15. Freire, J.R.J.; Kolling, J.; Vidor, C.; Pereira, J.S.; Kolling, I.; Mendes, N.C., - Sobrevivência e competição por sítio de nodulação de estirpes de *Rhizobium japonicum* na culture de soja. *Revista Brasileira de Ciência do Solo*, 7: 47-53, 1983
16. Johnson, H.W.; Means, U.M. - Selection of competitive strains of soybean nodulating bacteria. *Agronomy J.*, 56: 60-62, 1964.
17. Ledluy, A.; Zajic, J.E. - A geometrical approach for differentiation of an experimental function at a point: applied to growth and product formation. *Biotechnology and Bioengineering*, 15: 805-810, 1973.
18. Levean, J.Y. and Bouix, M. - Cineticas microbianas. In: *Biotechnologia* (Ed. SCRIBAN, R.), São Paulo, São Paulo, Brazil, 103-123, 1984.
19. Lopes, E.S,; Giardini, A.R.; Kilhl, R. and Igue, T. - Especificidade hospedeira e pré-seleção de estirpes de *Rhizobium japonicum* para as variedades Santa Rosa, Viçosa e IAC-2 de soja *Glycine max* (L.) Merril. *Bragantia*, Campinas, Brazil, 35: 1-12, 1976.
20. Lopreto, C.R.; Mazza, L.A.; Balatti, A.P. - Influencia de los componentes del medio de cultivo sobre el tiempo de "generacion" de una cepa de *Rhizobium japonicum*. *Anales de la Sociedad Cientifica Argentina*, 193: 35-47, 1972.
21. Lopreto, C.R.; Mazza, L.A.; Balatti, A.P. - Influencia del suministro de oxigeno sobre la velocidade de crecimento de una cepa de *Rhizobium japonicum*. *Rev. Asoc. Arg. Microbiol.*, 5: 128-131, 1973.
22. Lopreto, C.R.; Mazza, L.A.; Balatti, A.P., 1975. Estudos de crecimento de diferentes cepas de *Rhizobium japonicun*. *Revista de la Fac. Agron.*, La Plata, Argentina, 51: 35-41, 1975.
23. Mazza, L.A.; Lopreto, C.R. and Balatti, A.P. - Obtencion de cultivos de *Rhizobium japonicum* en un fermentador sin agitacion mecanica. *Rev. Asoc. Arg. Microbiol.*, 8: 99-103, 1976.
24. Murdock, D.I.,; Hatcher, W.S. - Growth of micro-organisms in chilled orange juice. *J. Milk Food Technol.*, 38: 393-396, 1975.
25. Oliveira, L.A. - *Eficiência, Capacidade Competitiva e Sobrevivêcia de Estiroes de Rhizobium japonicum (Kirchner) Buchmanan.* Masters Dissertation, Faculdade de Agronomia, UFRGS, Porto Alegre, Rio Grande do Sul, Brazil, 1982, pp. 142.
26. Pereira, J.C. -*Obtenção e Avaliação de Mutantes Espontâneos de Rhizobium phaseoli Resistentes a Antibióticos e Fungicidas.* Masters Dissertation, Faculdade de Agronomia, UFRGS, Porto Alegre, Rio Grande do Sul, Brazil, 1983, pp. 89.
27. Roughley, R.J.; Vincent, J.M. - Growth and survival of *Rhizobium* spp. in peat culture. *J. App. Bacteriol.*, 30: 362-375, 1967.
28. Skrdleta, V.; Karimova, J. - Competition between two somatic serotypes of *Rhizobium japonicum* used as double-strain inocula in varying proportions. *Arch. Microbiol.*, 66: 25-28, 1969.
29. Somasegaran, P.; Hoben, H.J. -*Methods in Legume-Rhizobium Technology.* Niftal Project (Document # 85:0301), Hawaii, U.S.A, 1985., 367p.
30. Specht, A.W.; Erman, L.W.; Means, V.M and Resnicky, J.W. - Effect of nutrition on *Rhizobium* hurtum inoculated with *Rhizobium trifolii*. *Soil Sci. Soc. Am. Proc.*, 29: 489-495, 1956.
31. Thiemann, J.E. - Estudios sobre a absorção de oxigêio em frascos agitados e em fermentadores experimentais. *Ciência e Culture*, 11: 173-189, 1959.
32. Trinick, M.J. - Competition between rhizobial strains for nodulation. In: Vincent, J.M., ed. -*Nitrogen Fixation in Legumes*, Academic Press, Sidney, Australia, 229-237, 1982.
33. Vincent, J.M. - Survival of the root-nodule bacteria. In: Hallsworth, E.G., ed. -*Nutrition of the Legumes*, Butterworth, London, U.K., 108-123, 1958.
34. Vincent, J.M. - Root-nodule symbiosis with *Rhizobium*. In: Quispel, A., ed. - *The Biology of Nitrogen Fixation, North Holland Research Monographs*, Amsterdam, Netherlands, 265-341 1974.
35. Vincent, J.M. - *Manual Práctico de Rhizobiologia*, Editoria Zaragosa, Brazil, 1975, pp. 200.
36. Vincent, J.M.; Waters, L.M. - The influence of the host on competition amongst clover root-nodule bacteria. *J. Gen. Microbiol.*, 9: 357-370, 1953.

Rev. Microbiol., São Paulo, **26**(3): 219-223, 1995

XYLANASE PRODUCTION BY *ASPERGILLUS* ISOLATES GROWN ON CORN COB

Marina K. Kadowaki[1]
Maria A. C. Pacheco[1]
Rosane M. Peralta[1]*

ABSTRACT

Production of xylanases (E.C. 3.2.1.8) by five *Aspergillus* soil isolates grown on corn cob as substrate was investigated. The isolates were apparently non-cellulolytic and may be utilized for applications in the pulping industry. The highest xylanolytic activities were obtained in cultures using 3-5% corn cob powder. The addition of 0.2% xylan or β-methylxyloside increased the production of xylanases. Carboxymethylcellulose was a weak inducer of both xylanase and β-xylosidase activities. The analysis of xylan hydrolysis by these system detected xylose as the main product.

Key words: xylanolytic fungi, cellulase-free xylanases, β-xylosidase, *Aspergillus*.

INTRODUCTION

β1,4 Xylans comprise up to 25% of hardwood biomass and are found in a variety of agricultural residues (7). They represent a potential renewable energy resource which could be utilized to improve the technology of bioconversion of plant biomass into useful products. The use of xylanases (E.C. 3.2.1.8, β1,4 D-xylan-xylano hydrolase) in conjunction with cellulases for the complete conversion of cellulosic biomass to sugars has been widely studied and shown to improve the overall economy of lignocellulosic biomass processing (4). Large quantities of xylan are released as effluents by the paper and pulp industries and their bioconversion may be of economic significance. Recently, the use of cellulase-free xylanases as a selective hydrolysis system for the removal of hemicellulose components in paper and pulp has been suggested (3,16,23,24).

The use of purified xylan as an inducer increases the cost of enzyme production and is the major limitation to the economic utilization of lignocellulosic materials. The purpose of this work was to isolate from soil filamentous fungi with the ability to produce cellulase-free xylanolytic enzymes on a cheap and abundant growth substrate (corn cob).

MATERIALS AND METHODS

Microorganisms: Fungi were isolated during a screening program for xylanase-producing microorganisms from soil. Screening was carried out using mineral medium (10) with 1% birchwood xylan (Sigma) as a carbon source. Isolates that showed a hydrolysis halo (at least 0.5 cm) on this medium were selected for later xylanase determination. The organisms were stored on potato

[1] Departamento de Bioquímica, Universidade Estadual de Maringá, 87020-900, Maringá, Pr - Brasil
* To whom correspondence should be addressed

dextrose agar slants (18) at 4°C. Spore suspensions were prepared by adding 10 mL of sterilized water to slant cultures and gently rubbing the surface with a sterilized wire loop.

Enzyme production in semi-solid culture: One mL of the spore suspensions (10^4 spores) was used to inoculate Erlenmeyer flasks containing 25 mL of Vogel salts (14) and 5 g of sterilized corn cob powder. After 5 days at 30°C, 50 mL of deionized water was added and stirred on a rotary shaker at 120 rpm for 30 min at room temperature. The medium was filtered (Whatman n° 1 filter paper), centrifuged at 3000 rpm for 15 minutes and the supernatant was used for determination of enzymatic activities.

Enzyme production in liquid culture: 10^4 spores were inoculated into 125 mL Erlenmeyer flasks containing 25 mL of Vogel salts and one of the following carbon sources at different concentrations: corn cob powder, birchwood xylan (Sigma) and carboxymethylcellulose (CMCellulose, Sigma). The cultures were incubated at 30°C on a rotary shaker at 120 rpm. After 5 days, the mycelium was removed from the culutre media by filtration.

Enzyme assays: Xylanase was assayed using birchwood xylan (Sigma) as substrate. One mL of the reaction mixture contained 0.5 mL of appropriately diluted enzyme solution and 0.5 mL of a 1% suspension of xylan in citrate-HCl buffer (0.05 M, pH 5.4). The mixture was incubated at 50°C for 10 min and the reducing sugars were assayed by the dinitrosalicylic acid procedure (12), with xylose as standard. The carboxymethylcellulolytic activity was assayed by a similar method, utilizing 1% CMCellulose as substrate in 0.05 M phosphate buffer (pH 6.0) and glucose as standard; the mixture was incubated for 60 min. In both cases, activities were expressed as moles of xylose or glucose per minute for each mL of medium. β-xylosidase activity was assayed by incubating 1 mL of 0.1% p-nitrophenyl-β-D-xylopiranoside (PNPX) with 0.1 mL of suitable diluted enzyme in 0.05 M phosphate buffer (pH 6.0) for 30 minutes at 50°C. The reaction was stopped by addition of 2 mL of 1 M Na_2CO_3. The liberated p-nitrophenol was measured spectrophotometrically at 410 nm.

Protein estimation: Extracellular protein was determined from TCA precipates (19) of culture filtrates using the method of Lowry *et al.* (9). Bovine serum albumin was used as standard.

Chromatography of xylan and cellulose hydrolysis products: The profile of birchwood xylan and cellulose (Avicel) enzymatic hydrolysates by several *Aspergillus* preparations was studied using paper chromatography. Enzymatic preparations obtained from liquid cultures containing 3% corn cob powder were dialysed overnight and incubated with 1% birchwood xylan or 1% cellulose at 50°C. After 10, 120 and 300 minutes, the reactions were heat inactivated and stored at 4°C until use. Samples (15μl) were spotted onto Whatman n° 1 filter paper and the hydrolysis products resolved by descendent chromatography at room temperature, utilizing a benzene:n-butanol:piridine:water solvent system (1:5:3:3) (8). The hydrolysis products were visualized with silver nitrate (20).

RESULTS

A total of 128 fungi were tested with respect to growth capacity at 28-30°C for up to 7 days in mineral medium containing xylan as the only carbon source. Colonies that grew well under such conditions, or cultures that showed a clear zone around the colonies, were selected and inoculated onto semisolid medium using 20% (w/v) corn cob powder as substrate. Since the aim of this work was to isolate fungi able to produce cellulase-free xylanolytic enzymes, we selected eight *Aspergillus*

TABLE 1. Production of xylanases, β-xylosidase and CMCase by Aspergillus isolates on semi-solid cultures supplemented with corn cob.

Strains	Enzymatic activity (U/ml) (a)			Protein
	xylanase	β-xylosidase	CMCase (b)	(mg/ml)
Aspergillus sp	37.1	3.01	0.048	0.52
A. flavus var 1	35.8	3.80	0.046	0.64
A. flavus var 2	58.4	4.58	0.052	0.86
A. parasiticus	54.1	2.14	0.059	0.68
A. niger var 1	47.9	5.17	0.055	0.57
A. niger var 2	35.5	4.04	0.057	0.67
A. tamarii	53.0	2.31	0.054	0.71
A. fumigatus	54.1	2.32	0.055	0.78

(a) The definitions of enzymatic activity are described in Materials and methods.
(b) CMCase = carboxymethylcellulase activity

TABLE2. Effect of different carbon sources on the production of xylanase,β-xylosidase and CMCase by Aspergillus isolates in liquid cultures (a)

Strain	Enzymatic Activity (U/ml)								
	Xylanase			β-xylosidase			CMCase		
	xylan	corn cob	CMC	xylan	corn cob	CMC	xylan	corn cob	CMC
A. flavus 2	50.8	41.1	2.02	0.93	2.32	0.32	0.06	0.05	0.06
A. parasiticus	51.7	34.1	4.06	0.85	1.80	0.19	0.04	0.04	0.03
A. niger 1	42.5	27.4	3.77	2.05	2.84	0.57	0.07	0.05	0.05
A. tamarii	42.3	37.1	1.44	1.04	1.63	0.71	0.05	0.06	0.06
A. fumigatus	40.4	32.4	6.08	1.02	0.93	0.67	0.04	0.05	0.05

(a) Concentration of carbon source was 1% (w/v)

soil isolates that produced the highest levels of xylanase (superior to 35 U/mL) and the lowest CMCase activities (lower than 0.06 U/mL) (TABLE 1). The five best xylanase producers were cultivated in liquid media with different carbon sources at 1% (xylan from birchwood, CMCellulose or corn cob powder). Liquid cultures containing xylan or corn cob produced the highest levels of xylanase and β-xylosidase. The lowest activities were observed in CMCellulose cultures (TABLE 2). CMCase activities were low in all cultures.

The effect of the initial corn cob concentration on the production of xylanase by *Aspergillus* strains was investigated (FIGURE 1). There was at least a 2-3 fold increase in xylanase production when the corn cob concentration in the medium increased from 1 to 3-5%. CMCase activities were below 0.06 U/mL. Based on these results, a corn cob concentration of 3% was used in all subsequent studies.

The addition of xylan and β-methylxyloside (βMX), a synthetic xylobiose analogue, had a positive effect on the production of xylanase (TABLE 3). Neither xylan nor βMX increased the CMCase production in all tested cultures.

The products of xylan and cellulose hydrolysis by enzymes produced by several fungi were studied by paper chromatography (FIGURE 2). A mixture

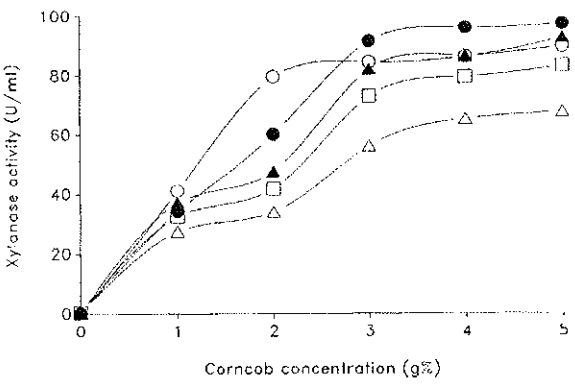


FIGURE 1. Effect of corn cob concentration in the production of xylanases (○) *A. flavus*; (●) *A. parasiticus*; (Δ) *A. niger*; (▲) *A. tamarii*; (□) *A. fumigatus*.

TABLE3. Effect of the addition of β-MX and xylan on Aspergillus cultures with 3% corn cob on the production of xylanase and CMCase (a)

Strain	added component	Enzymatic activity (U/ml)		protein (mg/ml)
		xylanase	CMCase	
A. flavus 2	--	79.1	0.06	0.98
	xylan	137.0	0.07	1.08
	βMX	184.3	0.05	0.88
A. parasiticus	--	91.5	0.06	1.30
	xylan	118.9	0.07	1.21
	βMX	168.7	0.07	1.35
A. niger 1	--	56.2	0.06	0.89
	xylan	108.4	0.04	0.75
	βMX	119.1	0.06	0.90
A. tamarii	--	82.3	0.06	0.75
	xylan	121.8	0.04	0.68
	βMX	169.5	0.05	0.71
A. fumigatus	--	73.9	0.07	1.35
	xylan	80.1	0.07	1.20
	βMX	77.9	0.08	1.43

(a) Xylan and βMX were utilized at 0.2% (w/v)

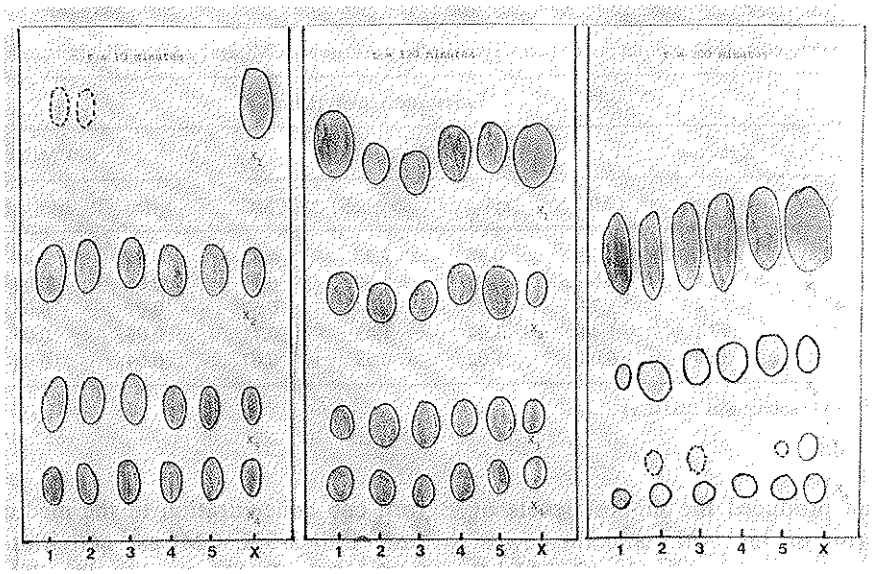


FIGURE 2. Diagrams of the paper chromatography of hydrolysates of 1,4 xylan by xylanolytic systems from *Aspergillus* strains. The reaction mixtures containing 0.5 mL of 1% xylan and 0.5 mL of xylanolytic enzymes were incubated at 50°C for 10, 120 and 300 minutes. Xylose (X_1) and xylooligosaccharides (X_2, X_3, X_4) are standards. 1. *A. flavus*; 2. *A. parasiticus*; 3. *A. niger*; 4. *A. tamarii*; 5. *A. fumigatus*.

of xylo-oligosaccharides was obtained after 10 minutes of hydrolysis. Xylose was detectable during the first ten minutes and continued to increase throughout the experimetal period. After 300 minutes of hydrolysis, the main product found was xylose. When cellulose was utilized as substrate for the hydrolysis process, glucose production was not detected (data not shown).

DISCUSSION

Different lignocellulosic sources like wheat straw, wheat bran, sugar cane bagasse and rice straw have been used as substrates to produce xylanolytic enzymes (5,6,22). At present, xylanases are obtained from processed or refined substrates such as sugars, cellulose and xylan. These substrates are relatively expensive for industrial scale production. Consequently, to lower the production costs, cheaper substrates for the manufacture of these enzymes are being sought. One of the possible substrates for xylanase production is corn cob. It is obtained with abundance and 42.5% of its chemical composition is hemicellulose (17). In this work, we obtained *Aspergillus* isolates able to produce cellulase-free xylanase in cheap media containing corn cob. When substrates were utilized at 1%, the best results were obtained with cultures containing xylan (TABLE 1). However, xylanase production raised with increasing corn cob concentrations (FIGURE 1). CMCellulose was a weak inducer of xylanases and -xylosidases (TABLE 2). Other studies have also shown that several fungi are able to produce xylanolytic activity when cultivated on cellulose (13,15). This may be caused by a non-specific activity of xylanases or the result of xylan contamination in cellulosic substrates (1,4).

The results obtained by adding 0.2% xylan or βMX to the corn cob media are in agreement with the data reported for other *Aspergillus* strains (1). On the other hand, no inducing effect by βM was observed with *Trichoderma longibrachiatum* (16).

The paper chromatography patterns of xylan hydrolysates by these xylanolytic systems (FIGURE 2) show the potential to attain a complete hydrolysis of xylan. The production of xylanases and β-xylosidases may be improved by an optimization of the medium and the culture conditions.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. D.S. Attili, Dr. M.A.Q. Cavalcanti and Dr. M.J.S. Fernandes for

the classification of the isolates used in this work, and to Dr. A. Bracht for his assistance in the preparation of the manuscript. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

RESUMO

Produção de xilanases por espécies de *Aspergillus* cultivados em sabugo de milho

A produção de xilanases (E.C. 3.2.1.8) por diferentes espécies de fungos pertencentes ao gênero *Aspergillus* isolados de solo foi investigada utilizando-se sabugo de milho como substrato. As espécies, aparentemente não celulolíticas, podem ser utilizadas na indústria de polpação. As maiores atividades xilanolíticas foram obtidas em culturas com sabugo de milho nas concentrações de 3 a 5%. A adição de xilano ouβ-metil-xilosídeo na concentração de 0,2%, aumentou a produção das enzimas. A análise dos produtos de hidrólise do xilano demonstrou que o principal produto formado foi xilose.

Palavras chave: fungos xilanolíticos, xilanases, β-xilosidase, *Aspergillus*.

REFERENCES

1. Bailey, M.J. and Poutanen, K. Production of xylanolytic enzymes by strains of *Aspergillus*. *Appl. Microbiol.*, 30:5-10, 1989.
2. Bailey, M.J.; Biely, P. and Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.*, 23:257-270, 1992.
3. Bajpai, P. and Bajpai, P.K. Biobleaching of kfraft pulp. *Process Biochem.*, 27:319-325, 1992.
4. Biely, P. Microbial xylanolytic systems. *Trends Biotechnol.*, 3:286-290, 1985.
5. Biswas, S.R.; Mishra, A.K. and Nanda, G. Xylanase and β-xylosidase production by *Aspergillus ochraceus* during growth on lignocelluloses. *Biotechnol. Bioeng.*, 31:613-616, 1988.
6. Chaudri, S.: Thakur, I.S.: Reeta, G. and Johri, B.N. Purification and characterization of two thermostable xylanases from *Melanocarpus albomyces*. *Biochem. Internat.*, 17(3):563-575, 1988.
7. Jeffries, T.W., *In*: Fiechter, A. (ed), *Advances in Biochemical Engineering/Biotechnology*, 27:1-32, 1983.
8. Gaillard, B.D.E. Use of unneutralized hydrolysates in paper chromatography of sugars. *Nature* (London), 171:1160, 1953.
9. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 13:265-275, 1951.
10. Mandels, M. and Weber, J. The production of cellulases. *Adv. Chem. Ser.*, 95:391-414, 1969.
11. Milagres, A.M.F. and Prade, R.A. Production of xylanases from *Penicillium janthinellum* and its use in the recovery of cellulosic textile fibers. *Enzyme Microb. Technol.*, 16:627-632, 1994.
12. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31:426-428, 1959.
13. Mishra, C.; Keskar, S. and Rao, M. Production and properties of extracellular endoxylanase from *Neurospora crassa*. Appl. *Environ. Microbiol.*, 48(1):224-228, 1984.
14. Montenecourt, B.S. and Eveileigh, D.E. Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Appl. Environ. Microbiol.*, 34:777-784, 1977.
15. Royer. J.C. and Nakas, J.P. Xylanase production by *Trichoderma longibrachiatum*. *Enzyme Microb. Technol.*, 11:405-410, 1989.
16. Royer, J.C. and Nakas, J.P. Interrelationship of xylanase induction and cellulase induction of *Trichoderma longibrachiatum*. *Appl. Environ. Microbiol.*, 56(8):2535-2539, 1990.
17. Singh, A. and Kalra, M.S. Single cell protein production from corn cobs. *J. of Food Sciences Technol.*, 15:249-255, 1978.
18. Smith, D. and Onions, A.H.S. (eds). The preservation and maintenance of living fungi. Commonwealth Mycological Institute, Kew, Surrey, U.K., 1983.
19. Tan, L.U.L.; Chan, M.K-H. and Saddler, J.N. A modification of the Lowry method for detecting protein in media containing lignocellulosic substrates. *Biotechnol. Lett.*, 6(3):199-204, 1984.
20. Trevelyn, W.; Procter, P.D. and Harrison, J.S. Detection of sugars on paper chromatograms. *Nature* (London), 166:444-445, 1950.
21. Yasui, T.; Nguyen, B.T. and Nakanishi, K. Inducers for xylanase production by *Cryptococcus flavus*. *J. Ferment. Technol.*, 62:353-359, 1984.
22. Warzywoda, W.; Ferre, V. and Pourquie, J. Development of a culture medium for large-scale production of cellulolytic enzymes by *Trichoderma reesei*. *Biotechnol. Bioeng.*, 25:3005-3011, 1983.
23. Wong, K.K.Y.; Tan, L.U.L. and Saddler, J.N. Multiplicity of beta 1,4 xylanase in microorganisms. Function and application. *Microbiol. Rev.*, 52:305-317, 1988.
24. Wong, K.K.Y. and Saddler, J.N. *Trichoderma* xylanases, their properties and application. *Crit. Rev. Biotechnol.*, 12:413-435, 1992.

Rev. Microbiol., São Paulo, **26**(3): 224-229, 1995

MICROBIOLOGICAL EVALUATION OF BUTTER PURCHASED FROM THE MARKET OF RIO DE JANEIRO. I. INDICATOR AND PATHOGENIC MICRORGANISMS

Elzabete G. Cavalcante dos Santos
Sonia Maria da Costa Raimundo
Paschoal Guimarães Robbs

ABSTRACT

The microbiological quality of 216 butter samples from 11 different brands sold in the State of Rio de Janeiro was evaluated. One hundred and thirty six samples belonged to the "extra-type", 60 were of "first quality" and 20 of the "common type". Counts were determined for total coliforms, fecal coliforms, enterococci, *S. aureus* and *Salmonella sp.* Total coliforms, fecal coliforms, enterococci and *S. aureus* were found in 56.9%, 37.5%, 100%, and 9.7% of the samples, respectively, whereas *Salmonella* was undetected in all cases. Nineteen percent of the samples did not conform to the Brazilian microbiological standard set by the Ministry of Health.

Key words: butter, pathogenic microrganisms, indicator microrganisms.

INTRODUCTION

Butter is not considered a perishable foodstuff, yet it can be modified by the action of bacteria and fungi. Cream constitutes the main source of microbial contamination, since most bacteria are carried by fat globules upon separation from whole milk (11). Foodborne pathogens are likely to be found among the contaminants, *Salmonella* sp. and *Staphylococcus aureus* being important dangerous species for this type of dairy product. *Salmonella sp.*may cause severe gastrointestinal infections in humans, especially in children and the elderly. In butter, the presence of *S.aureus* is caused by handling, underprocessing of raw material or by improper cleaning and sanitation of tools and equipment. In raw milk, mastitis is a major source of contamination with *S. aureus.* When high levels of contamination are present, part of the bacteria may resist pasteurization (5) and appear in pasteurized milk and dairy products.

Given the difficulty to isolate pathogens from food samples, certain groups of microorganisms more easy to enumerate are used as indicators of the sanitary quality of foods, pointing to the possible presence of dangerous species. Among such indicators are the total and fecal coliforms, *Escherichia coli* and the enterococci. These microrganisms, especially the coliforms, are rarely found in properly processed foods; their detection is a proof of failure in thermal processing and/or of unsatisfactory hygienization and sanitation procedures in the industry. *E. coli* is the best indicator of fecal contamination, evidencing the possible presence of enteric pathogens (10).

Studies on the microbiological profile of butter carried out in Brazil (4,15,17) and abroad (13,16,19) have demonstrated the presence of coliforms, *E. coli*, enterococci, *Salmonella* and/or *S. aureus* in the product .

Departamento de Tecnologia de Alimentos, Universidade Federal Rural do Rio de Janeiro, 23.851-970, Seropédica - RJ.

The present study aimed at evaluating the microbiological conditions of selected types of butter commercialy available in supermarkets of the State of Rio de Janeiro, with respect to the levels of contamination with indicator species and with some potentially pathogenic microorganisms. This study is part of a broad program developed to subsidize the establishment of microbiological specifications and/or standard for dairy products.

MATERIALS AND METHODS

Sampling

A total of 216 samples of butter from 11 different brands was examined. The samples included salted and unsalted varieties and the "extra", "first quality" and "common" types. Samples coded A, B, E, F, G, I and L were of the "extra" type (salted or unsalted); C, D, and H were of the "first quality" type (salted) and J corresponded to the salted "common" type. Brand L was withdrawn from the market during the experiments, thus reducing the samples analyzed to 16. Samples were collected in supermarkets of the State of Rio de Janeiro. All were within the validity period and properly packaged.

Five samples of different brands were collected weekly, taken to the lab inside insulated containers with ice and kept under refrigeration (4 - 7°C) for a maximum of 3 days before use for evaluation.

Dilutions

A 1/10 dilution was obtained according to Harper *et al* (10) by addition of 11 g of the solid samples (including aqueous and fatty phases) to 99 mL of phosphate buffer (pH 7,2) at 40°C. The samples were maintained in a water-bath at 40°C until complete melting, phase separation being avoided. This initial dilution was then used for further decimal dilutions.

Microbiological analysis

The following microbial counts and/or detections were performed, according to specialized methodology (3,10,12):

a) Total and fecal coliforms: evaluated by the most probable number (MPN) technique using three test tubes per dilution with lauryl sulfate trytose broth (incubation at 35°C/24-48h) for the presumptive test. Brilliant green lactose bile broth (2%) and EC broth were used for total and fecal coliform counts, incubation being carried out at 35°C/24-48h, and 44.5 + 0.2°C/24h, respectively.

b) Enterococci: evaluated by the pour plate method. One mL of the dilutions was inoculated onto plates containing KF Streptococcal agar medium supplemented with 0.1% 2-3-5 triphenyltetrazolium chloride, and incubated at 35°C for 48h.

c) *Staphylococcus aureus*: detection and count performed by enrichment and direct plating techniques. For detection, 1.0g of sample was added to 10mL of *Staphylococcus* enrichment broth supplemented with 0.6mL of potassium tellurite solution (3.5%) per 100mL of medium (incubation at 37°C/24-48h). Tubes with black color were considered positive. Confirmation was carried out by streaking onto Baird-Parker agar plates. Count plates were prepared by direct plating of 0.1 mL of each dilution (in duplicates) onto a surface of Baird-Parker agar. All plates were incubated at 37°C for 24-48h. Typical colonies were tested for coagulase by enrichment in brain heart infusion broth (incubation at 35°C/24h). Subsequently, 0.1mL of enrichment was added to 0.3mL of coagulase plasma and incubated at 37°C for 4-24h. Tubes with defined coagulation were considered positive.

d) Detection of *Salmonella*: this was done by non-selective enrichment of 25 g of each sample in 225 mL of lactose broth (incubation at 35°C/24h) followed by selective enrichment in tetrathionate broth and selenite cystine broth (incubation at 35°C/18-24h) and streaking of selective broths on brilliant green agar and SS agar plates (incubation at 35°C/24h). Typical colonies were further tested on triple sugar iron agar (TSI) and lysine iron agar (LIA).

RESULTS AND DISCUSSION

The microbiological examination of 216 butter samples purchased from supermarkets in Rio de Janeiro resulted in the percentage count distribution of quality indicators and pathogenic microorganisms presented in TABLE 1.

TABLE 1. Contamination levels and percentage distribution of total and faecal coliform, enterococci and S. *aureus* in 216 samples of butter obtained from supermarkets in Rio de Janeiro.

Microrganisms	Contamination Level								
(MPN/g or CFU/g)	≤0.3	0.3 - 3	3 - 10 (or≤10)	10 - 10^2	10^2 - 10^3	10^3 - 10^4	10^4 - 10^5	10^5 - 10^6	10^6 - 10^7
Total Coliforms	43.1	6.5	13.9	6.9	11.1	11.1	6.0	0.9	0.5
Faecal Coliforms	62.5	10.7	9.7	6.9	4.6	3.7	1.4	0.5	-
Enterococci	-	-	45.8	11.6	13.4	13.4	15.3	0.5	-
Staphylococcus aureus	-	-	90.3	6.9*	1.9	0.9	-	-	-

* 6.4 below 5 x 10 CFU/g

In Brazil, microbiological standards for foods available to consumers are established by Ordinance nr. 1, published in January 28th, 1987, by the National Division for Sanitary Vigilance of Foods (DINAL) of the Ministry of Health (6). According to microbiological counts, the samples can be classified as: Acceptable, Unsatisfactory, Unacceptable and Improper. Such ordinance does not state limits for either total coliforms or enterococci. However, there are maximum count limits for these microorganisms proposed by some authors (6,10,14,17). These will be discussed in relation to the data obtained in the present study.

Results for total coliforms (TABLE 1) indicate the counts were higher than 10/g (the most frequently recommended standard) in 36.5% of the samples.

Regarding (TABLE 1), 82.9% of the samples fell within the limits of the Ministry of Health standards while 6.9% presented unsatisfactory hygienic conditions, 4.6% were unacceptable for direct consumption and 5.6% improper for any kind of consumption. Therefore, 17.1% of the samples did not conform to faecal coliforms standards.

With respect to enterococci counts, standards are scarce. However, this group is recommended as indicator for heat-treated foods, since it is more heat-resistant than the coliforms (10). Within the recommended limits, the most tolerant standard was proposed by Oliveira (14), with a maximum count of 10^3 CFU/g. The data obtained in this study (TABLE 1) show that 29.2% of the samples analyzed were above this limit.

Isolation of *S.aureus* from commercial butter samples and the onset of foodborne diseases caused by *S.aureus* associated with butter consumption have been reported by several authors (13,16). In this investigation, counts for *S.aureus* (TABLE 1) were very satisfactory, with 96.7% of samples conforming to the Brazilian Standad (maximum of 50/g).

Salmonella sp. was not detected in any of the samples examined. In Spain, the presence of *Salmonella* was reported in 7% of the butter samples analized by Molina (13).

The percentage of samples falling out of the Ministry of Health standards was 19.0%. Faecal coliforms and *S. aureus* were responsible for the problems detected.

The quality observed in 40 egyptian butter samples (2) was worse than that presently recorded in Brazil (TABLE 1). High counts for total and faecal coliforms were found in 77.5% and 65% of the samples, respectively. In Iraq, counts higher than 10^2 faecal coliforms per gram where present in 67.7% of 30 butter samples analysed (1).

Considering the percentage of samples with contamination levels past those recommended for total and faecal coliforms (36.5% and 17.1%, respectively), the quality results obtained are worse than those reported abroad (13,16,19). The maximum percentage of samples out of standard in other countries is around 30% and is lower than those reported in some previous Brazilian studies (15,17), which ranged from 45 to 50%.

With respect to enterococci counts, the present results (TABLE 1) were better than those reported for Egyptian butters, were 72.2% of the samples

had more than 10^3 enterococci per gram (2). In the Egyptian study, an average of 1.3×10^4 enterococci/g was observed in 14 out of 16 samples (7).

Results obtained for staphylococci were better than those described by Faraone (8) in Italy, who found that, of a total of 76 samples, 46.2% were out of standards.

In Nigeria (20), counts for *S. aureus* in 79 butter samples varied from 3.4×10^2 to 2.2×10^3 CFU/g, indicating contamination levels higher than those for the Brazilian samples, where the maximum count was 10 CFU/g. This lower level of contamination is not sufficient to causea direct health problem yet it can lead to spreading of the microorganism when butter is used in the preparation of meals.

As shown in TABLE 2, brands A, C, D, G, and H exhibited good microbiological quality and all units were adequate for consumption. Brand B can also be included in this cathegory, although one sample had a high level of contamination with *S.aureus* and was classified as improper for consumption (data not shown). According to Ordinance 01/87, brands J and L can be classified as "regular", because some samples were considered improper for direct consumption and presented unsatisfactory hygienic conditions. Brands E, F and I can be classified as "poor quality" due to their low percentage of acceptable samples (45%, 65% and 50%, respectively). Brands E and I were the least satisfactory, since a high percentage of their samples was classified as improper for consuption (25% and 30%, respectively).

Concerning the type of butter, it should be mentioned that the classification into types depends on the heat treatment and acidity levels of the cream used for production. Therefore, cream for "extra" and "first quality" types must be pasteurized, with maximum acidity values of 35°D and 50°D, respectively. For the "common" type, the acidity can be as high as 65°D, and pasteurization is not required.

Conditions of facilities and equipment are more stringent for industries producing "extra" and "first quality" types. Therefore, a difference in microbiological quality is expected between types. In the present study, 136 butter samples of the "extra" type, 60 of "first quality" and 20 of the "common" type were examined. Results are summarized in TABLE 3 and show the percentage of samples within the proposed limits. Unexpectedly, "first quality" butters presented better results than "extra" type ones. The lower quality of "common" butters was confirmed.

No distinction is made between salted and unsalted butters by the Brazilian standards regarding microbiological counts. Nevertheless, a comparison was made between samples of 5 brands, using a total of 50 samples for each variety. TABLE 3 gives the percentage of samples within the limits for microorganisms more frequently suggested or used as reference. With respect to the indicators of

TABLE 2. Percentage of samples examined per butter brand, for each stated limit of contamination.

Microrganisms	Butter Brands										
	A	B	C	D	E	F	G	H	I	J	L
Enterococci (< 1 x 10^3 CFU/g)	100	75	95	60	30	80	85	100	40	45	69
Total Coliforms (< 1 x 10 MPN/g)	100	100	70	75	15	60	75	95	20	45	44
Faecal Coliforms (< 1 x 10 MPN/g)	100	100	95	90	50	65	95	95	55	80	88
S. aureus (<1 x 10 CFU/g)	100	95	100	100	95	95	100	100	85	100	94
Salmonella (absence/25g)	100	100	100	100	100	100	100	100	100	100	100

TABLE 3. Classification by type and variety of 216 samples of butter obtaind from supermarkets in Rio de Janeiro, according to stated limits (results are given as percentage values).

Microrganisms	Limit Considered	Type (Number of Samples)			Variety (Number of Samples)	
		Extra (136)	First quality (60)	Common (20)	Salted (50)	Unsalted (50)
Enterococci	$<1x10^3$ CFU/g	68	85	45	70	60
Total coliforms	< 10/g	59	80	45	40	44
Faecal coliforms	< 10/g	79	93	80	80	68
S. aureus	< 5x10 CFU/g	95	100	100	96	92
Salmonella	absence/25g	100	100	100	100	100

hygienic conditions and to *S. aureus*, no remarkable difference can be noticed between the two varieties. These results are in accordance with the data obtained by Shehata *et al* (18) in Cairo, in a study on butters produced with pasteurized sweet cream and containing different salt levels. The authors concluded that NaCl has no definite influence on the development, incidence and distribution of microorganisms in butter samples stored at 0°C/90 days .

RESUMO

Avaliação microbiológica de manteigas comercializadas no Rio de Janeiro. I. Microrganismos indicadores e patogênicos

Foram examinadas, com relação aos microrganismos indicadores de condições higiênico-sanitárias e aos potencialmente patogênicos, 216 amostras de manteiga pertencentes a 11 diferentes marcas comercializadas no Estado do Rio de Janeiro, sendo 136 do tipo extra, 60 de 1ª. qualidade e 20 do tipo comum. Foram feitas contagens de coliformes totais, fecais, de enterococos e de *Staphylococcus aureus* e a pesquisa de *Salmonella*. 56,9% das amostras foram positivas para coliformes totais; 37,5% para coliformes fecais; 100% para enterococos e 9,7% para *Staphylococcus aureus*. *Salmonella* não foi detectada em nenhuma das amostras. 19,0% das amostras estavam fora dos padrões microbiológicos estabelecidos pelo Ministério da Saúde, Brasil.

Palavras-chave: manteiga, microrganismos patogênicos, microganismos indicadores, saúde pública.

REFERENCES

1. Abbar, F.M.; Mohamed, M.T. Ocurrence of enteropathogenic *E.coli* sorotypes in butter. *J. Food Protec.* 50(10):829-831, 1987.
2. Ahmed, A.A.H.; Moustafa, M.K.; Abdel-Hakien, E.H. Sanitary conditions of cooking butter manufactured in Assint city. *Assint Vet. Med. J.* 19(37):81-86, 1987.
3. American Public Health Association (APHA). *Compendium of methods for Microbiological Examination of Foods*. Marvin L. Speck (ed.) Washington, 1984, p.914.
4. Brum, M.A. Pesquisa de agentes microbiológicos que mais frequentemente determinam alterações na manteiga durante a conservação. *Rev. Inst. Lat. Cândido Tostes.* 29:11-19, 1974.
5. Dabbah, R.; Moats, W. A.; Edwards, U. M. Heat survival curves of food - borne bacteria suspended in commercially sterilized whole milk. II. Bacteria other than Salmonellae. *J. Dairy Sci.* 54:1772-1779, 1971.
6. Divisão Nacional de Vigilância Sanitária de Alimentos (DINAL). Portaria nº 01, de 28 de janeiro de 1987. Secretaria Nacional de Vigilância Sanitária, Ministério da Saúde, Brasília, 1987.
7. EL-Bassiony, T. A. Enterococci in some dairy products. *Assint Vet. Med. J.* 15(29):107-111, 1985.
8. Faraone, P. Pathogenic staphylococci in milk products. Mondo Latte 20(7):539-542. 1966 *In*: Dairy Sci. Abst. 19(1):44, 1967.
9. Halpin-Dohnalek, M.I.; Marth, E.H. Fate of *Staphylococcus aureus* in whipped butter. *J. Food Protec.* 52(12):863-866, 1989.
10. Harper, C. JR.; Dizikes, J. L.; Pachard, V.S. Microbiological methods for butter, margarine and related products. *In*: Standard Methods for Examination of Dairy Products. Marth, E. H. ed., 14ª. ed. Washingtion, Am. Public Health Assoc., 1978, p.157-159.
11. Jay, J.M. Microbiologia Moderna de los Alimentos. Zaragoza, Acribia, 1978, 491 p.
12. Laboratório Nacional de Referência Nacional (LANARA). *Métodos de Análises Oficiais para Controle de Produtos de Origem Animal e seus Ingredientes*. Métodos Microbiológicos. Ministério da Agricultura, Secretaria Nacional de Defesa Agropecuária, 1981.
13. Molina, J.S. Calidad microbiológica de mantequillas espanolas. *Arch. Zoot.* 26(103):241-245. 1977.
14. Oliveira, C.C. *Qualidade microbiológica da manteiga a nível de indústria*. Tese de Mestrado. Universidade Federal Rural do Rio de Janeiro - RJ. 1984, 133 p..
15. Reis Filho, S.A.; Iaria, S.T. Microrganismos em manteigas

vendidas no Município de São Paulo. *R. Saúde Púb.* 15:418-433, 1981.

16. Rose, C. de La; Mosso, A.; Mohino, M.; Gaston de Tricarte, E. Microbiological quality of pasteurized Spanish butter. *An. Bromat.* 30(3-4):333-338, 1978.
17. Sandoval, L. A.; Paulo, M.S.; Killner, M. Estudo tecnológico e microbiológico de manteigas consumidas no Estado de São Paulo. *R. Inst. Lat. Candido Tostes.* 156-157:1-3, 1971.
18. Shehata, A.E.; Magdoub, M.N.I.; El-Saragy, Y.A.A.; Hassan, A.A. *Effect of salt grade on the microbiological quality of butter.* Egyptian J. Dairy Sci. 5(2):97-103, 1977. *In:* Food Sc. Tech. Abst. 10(8):1198, 1978.
19. Ubach Turul, M. Microbiological quality of Spanish butter and margarines. *Ann. Bromat.* 37(2):307-313, 1986.
20. Umoh, V.J.; Adesiyn, A.A.; GomwalK, N.E. The occurrence of *Staphylococcus aureus* in fermented milk products (Fura and Manshanu) in Nigeria. *Inter. J. Food Microb.*, 10(3/4):343-348, 1990.

Rev. Microbiol., São Paulo, **26**(3): 230-235, 1995

UTILIZATION OF PHOSPHINE FUMIGANT FOR THE CONTROL OF FUNGI NATURALLY PRESENT IN STORED PADDY RICE (*ORYZA SATIVA*, L.)

Maria Fernanda P.P.M. de Castro[1]
Ivânia Athié Pacheco

ABSTRACT

Freshly harvested paddy rice, with initial water activities (a_w) of 0.84 and 0.90 (16.0% and 19.1% moisture content on a wet basis, respectively), was exposed to 0.0; 0.5; 1.0 and 1.5mg phosphine (PH_3)/L at 28°C for 7 and 14 days. Fumigant concentrations were determined after each exposure period. Fungal flora was evaluated before and after all treatments by direct plating onto two media. The results showed a decrease of total infected grains as the fumigant concentration increased. After all treatments, a better control was observed for field fungi at 92% relative humidity and for storage fungi at 85% relative humidity. The 0.5mg PH_3/L concentration was probably sufficient for the control of *Eurotium* spp development at both relative humidities. The results suggest that the 1.0 and 1.5mg PH_3/L concentrations were effective for controlling storage fungi after the two exposure periods at both relative humidities. The lowest percentage of fungal infection of grains was obtained with 1.5 mg phosphine/L at the highest relative humidity. The protection period offered by the fumigant depended on the concentration applied.

Key words: paddy rice storage, phosphine, control of fungi.

INTRODUCTION

In many rice producing regions, the product retains a high moisture content for some days after harvest, due to the absence of infrastructure and/or adequate weather conditions for grain drying. Such situation favours the development of fungi that may cause dry matter losses and commercial depreciation of the agricultural commodity due to discoloration and/or production of micotoxins, rendering the grain unfit for human consumption.

The fumigant phosphine, widely applied for insect control during storage, has been shown to have an effect on fungi present in sorghum, wheat and rice. Raghunatan *et al.* (13) and Sinha *et al.* (14) verified only a minor influence on now-growing moulds; Hocking and Banks (7), using phosphine at a dose of 0.1mg/L observed a decrease in fungal development on wheat at 0.80 and 0.86 water activities during storage for two weeks at 28°C. Hocking and Banks (8) also detected a reduced proliferation of *Aspergillus parasiticus* inoculated on paddy rice at 0.92 a_w after treating the grain with 0.1mg/ $PH_{3/L}$ for 14 and 28 days; it was found that phosphine decreased the rate of development of most storage fungi with the exception of *Penicillium* sp. These authors suggested that higher fumigant concentrations should be tested. Castro *et al.* (4), using 0.5, 1.0 and 1.5mg phosphine/L on shelled peanuts stored with a high moisture content (0.89

[1] Corresponding author: Instituto de Tecnologia de Alimentos - ITAL - Av. Brasil, 2880, Caixa Postal, 329 CEP 13073-001 - Campinas, SP, Brazil

a_w, equivalent to 16.5% moisture content on a wet basis), observed that the lowest fumigant concentration was sufficient to control B_1 and G_1 aflatoxin production for up to 14 days of storage. The reduced CO_2 yield obtained during phosphine treatment indicated that fungal growth was probably inhibited at higher fumigant concentrations and delayed at the lowest dose applied during the storage period of 14 days.

The aims of the present work were: a) to verify the effects of different phosphine concentrations on the control of field and storage fungi in paddy rice and b) to analyse the effect of phosphine on the microflora of paddy rice with a high moisture content exposed to the fumigant for different periods of time.

MATERIALS AND METHODS

Freshly harvested paddy rice (*Oryzae sativa* L.) of the IAC-25 variety, derived from the 1992 crop produced at the Experimental Station of the Instituto Agronomico in Mococa-SP, was used as test material. The sample was divided into two lots: one was sun-dried to reach a moisture content of approximately 16.0% on a wet basis (0.84 a_w); the other was used at the harvesting moisture content of 19.1% on a wet basis, (0.90 a_w). Water activity values were determined in a Novasina AG model EEJA/3 BAG linked to a conditioned chamber. Triplicate determinations of moisture content (m.c.) were made by placing 15g amounts of rice in a forced convection oven at 103°C 3°C for 72 hours (1). For each moisture content used, a lot was manually homogenized and further divided into 16 sub-samples of 250g each. The sub-samples were then placed onto petri dishes and distributed inside dessicators with a capacity of 9 L.

The dessicators were adapted for phosphine application using a silicone tube (approximatelly 50mm in length and 3.0mm in diameter) which had one end fixed to an orifice made in the lid by using silicone rubber. The eventual sealing of the tube was made with a staple and by the application of silicone rubber to its end.

To keep the samples with initial 0.90 and 0.84 a_w atmospheres of 92% and 85% were established according to Wiston and Bates (15) by placing 0.5 l per dessicator of saturated sodium tartarate and potassium chloride solutions, respectively; eight dessicators of each solution were prepared in this manner. Phosphine was applied at 0.0; 0.5; 1.0 and 1.5mg/L concentrations; the dessicators were maintained for 7 and 14 days inside a chamber at a controlled temperature of 28°C. Two repetitions were carried out for each phosphine treatment and period of exposition.

Phosphine was obtained in a generator according to the FAO methodology (5) using 0.6g of aluminium phosphide pills; the gas concentration in the generator was determined by a conductimetric method (6) based on the phosphine mercurium chloride reaction. The volume of phosphine necessary to produce each testing concentration of fumigant inside a dessicator was calculated and the gas applied with appropriated syringes.

Fumigant concentrations inside the dessicators after each exposure period (7 and 14 days) were evaluated by the conductimetric method previously mentioned.

Samples from each dessicator were homogenized manually and a sub-sample of approximately 20g removed for mycological analyses.

The direct plating technique for detection of fungi was employed. Grains were immersed in a 2% hypochloride solution for three minutes and then rinsed three times with sterile distilled water. Fifty grains were disposed onto five Petri dishes (10 grains/plate) containing the culture media DRBC (Dichloran Rose Bengal Chloraphenicol Agar) (10) or DG18 (Dichloran 18% Glicerol Agar) (9). Plates were incubated in B.O.D. at 25°C and in the dark, for five (DRBC agar) or seven (DG18 agar) days. Developed colonies were quantified by stereoscopic microscopy and isolated on apropriated media for further identification. Data are presented as the percentage of grains contaminated by each specified fungal species .Some of the grains showed more than one colony of the same specie or of different species.

RESULTS AND DISCUSSION

1. Phosphine concentration

Average final phosphine (PH_3) concentrations after exposure of paddy rice to different doses of the fumigant at 85% and 92% RH for 7 and 14 days are shown in TABLE 1.

TABLE 1. Final PH_3 concentrations (average of two experiments) in environments at 85% and 92% R.H., 7 and 14 days after applications of different initial concentrations of the fumigant.

PHs Concentrations (mg/l)				
initial	final			
	7 days		14 days	
	85%	92%	85%	92%
0,00	0,00	0,00	0,00	0,00
0,50	0,08	0,06	0,05	0,04
1,00	0,25	0,18	0,10	0,07
1,50	0,43	0,49	0,17	0,13

Differences between initial and final concentrations of phosphine for the two exposure periods and the two relative humidities (TABLE 1) are probably related to adsorption of the fumigant by the product, as expected. For each exposure period, a tendency towards higher fumigant adsorption at 92% R.H. compared to 85% R.H. was observed. It was also recorded that most of the fumigant was adsorved during the first 7 days of exposure (77% and 79%, on average, at relative humidities of 85% and 92%, respectively); after 14 days, the total adsorption values of the phosphine applied were 90% at 85% R.H. and 92% at 92% R.H. The high adsorption rates of phosphine observed in this work confirm the comment made by Annis (2) that paddy rice seems to show a high and variable adsorption, particularly when fumigated for the first time or freshly harvested.

2. Analysis of the mycoflora

2.1. Samples kept at 85% Relative Humidity

Samples kept at 85% relative humidity and plated onto DRBC agar revealed that 92% of the grains were contaminated. The predominant micoflora comprised mainly pigmented and non-pigmented yeasts (74%) and *Phoma* spp(64%). Other non-sporulating fungi (38%), *Cladosporium* spp (4%) and *Fusarium* spp (2%) were also detected. However when using DG18 agar, a more appropriate medium for the detection of xerofilic species, 96% of the grains were contaminated ,the ocurrence of *Phoma* spp was verified in 84% of the grains. The presence of *Cladosporium* spp (6%), *Fusarium* spp (2%) and others (6%) was also observed. These fungi normally colonize the grains while the plants are growing in the field or just before harvest, and are known as field fungi.

After 7 days storage, the DG18 agar-cultures showed that samples not exposed to the fumigant (control samples) were also contaminated with *Eurotium amstelodami* (2%) and *E. chevalieri* (1%) sp and *Penicillium citrinum* (1%). After 14 days of storage, a decrease of field fungi could be observed in DG18 agar cultures (from 97% to 78%), as expected, probably due to conditions less favorable for the growth of this group of fungi . Concomitantly, also as expected, there was a marked increase in the number of grains infected with *Eurotium* spp (from 3% to 76%), predominantly *Eurotium amstelodami* (from 2% to 73%); *A. nidulans* was also detected (1%) . This marked increase of *Eurotium* spp could not be verified on DRBC agar, as this medium is not appropriate for the detection of xerofilic species. However, *Eurotium* spp were no longer detected in samples treated with phosphine at the three doses studied, thus indicating that these species could undergo physiological damaged by treatment with the fumigant, as suggested by Hocking and Banks (7). The control of *Eurotium* spp is of great interest since these xerofilic fungi are frequently the primary invaders of stored grains (Pitt and Hocking, 1985; cited by Hocking and Banks) (8) and their metabolism, once the invasion has taken place, increases grain moisture content and allows the additional establishment and growth of potencially micotoxigenic fungi, such as *A.flavus* and *A.parasiticus* (8). *Penicillium* species, identified as *P.brevicompactum* and *P.chrysogenum* which are considered non-toxigrnic were still detected on both media at non-significant percentages after 14 days of treatment with 0,5mg PH_3/L (2% of *P. brevicompactum* on DRBC agar) and 1,0mg PH_3/L (1% of *P. brevicompactum* on DRBC and DG18) and also after 7 days of application of the highest phosphine concentration (1% of *P.chrysogenum* on DRBC agar). Conditions of a_w<0.90 are, in general, less favorable for the development of *Penicillium* species, indicating that their presence, although at irrelevant levels, could be due to competition with other species which were controlled by the fumigant or to a higher tolerance of this genus to phosphine, as suggested by Hocking and Banks (7).

2.2. Samples kept at 92% Relative Humidity

Rice kept at 92% relative humidity on DRBC agar also showed an initial high percentage of

grains internally infected (94%), exhibiting mainly pigmented and non-pigmented yeasts (80%),*Phoma* spp (56%), *Cladosporiun* spp (4%) and other non-sporulating field fungi (40%) . When using DG18 as culture medium, it was observed that 98% of the grains were infected with fungi, predominantly with *Phoma* spp (76%), *Cladosporium* spp (14%), *Fusarium* spp (4%) and others (8%) .

After 7 days of storage, the more relevant changes were observed on DG18 agar. In the non-treated samples, 21% of the grains were infected with storage fungi and 14% exhibited *Eurotium* spp , the predominant species being *E. amstelodami* (13%) .*A. nidulans* (3%), *A. niger* (1%), *A. penicilloides* (1%) and *Penicillium* spp (1% of *P. chrysogenum*) were also detected . After 14 days of storage, an increase in the percentage of grains infected with *Eurotium* spp was observed (from 14% to 42%), which was lower than the one verified at 85% R.H. (from 3% to 76%), a R.H. level more favourable to *Eurotium* species. A small increase from 1% (*P. chrysogenum*) to 5% (4% of *Penicillium chrysogenum* and 1% of *P. citrinum*) after 14 days at 92% R.H was also observed, as this condition generally favours the development of *Penicillium* species more than 85% R.H. . When using DRBC agar, no relevant changes were observed at the end of both exposition periods (7, 14 days). In the samples treated with 0.5mg/L of phosphine, no *Eurotium* spp was observed on DG18 agar 7 days after fumigant application . The control of these species might have favoured the slight increase of *A. nidulans* from 1% to 4% (DRBC agar) and from 3% to 7% (DG18 agar) . This situation was more evident after 14 days, when an increase of *A. nidulans* from 4% to 28% on DRBC medium and from 7% to 43% on DG18 medium was detected . This observation clearly indicates the need to establish adequate fumigant concentrations for an efficient control, as an insufficient dose may decrease species competition and favour the development of potentially toxigenic fungi (such as *A. nidulans*). The evaluation on DG18 agar indicates that the development of *A. versicolor*, that showed an increase from 2% to 7%, and of *Penicillium* species, that showed an increase from 1% (*Penicillium* sp) to 6% (*P. chrysogenum*), also seems to have been favoured . However, these species were not detected on both culture media after exposure for 7 days to 1.0 mg PH_3/L. The reappearance of these fungi 14 days after the application of 1.0mg PH_3/1 was probably favoured by the significant decrease of field fungi, thus reducing inter-species competition. Another possibility is that higher concentrations are needed for control during a more extended period of time, as *A. nidulans* and *A. versicolor* were not observed after 14 days of application of 1.5mg PH_3/L . The need to use higher fumigant concentrations associated to an extended exposure period was also verified by Castro *et al.*. (4).

The absence of *Eurotium* spp at the 85% R.H. and the ocurrence of these essencially xerofilic species at the 92% R.H., althoug in insignificant percentages, at the 0.5; 1.0 and 1.5mg/L phosphine concentrations , might be due to a more effective phosphine action of the fumigant under conditions more favourable to the development of these fungi. However, a reduced competition between *Eurotium* spp and field fungi at 92% R.H., which were efficently controled at these phosphine concentrations, canot be ruleed out. Only 2% of *Penicillium* spp (*P. brevicompactum*) and 1% of the *Eurotium* spp could be detected at the higher concentration for the longer exposition period (DG18 agar) . The presence of *Penicillium* spp may indicated that this genus is more resistant than the others, as related by Hocking and Banks (7).

2.3. General considerations

In general, results indicate a gradual control of the percentage of grains infected as the concentration of fumigant applied increases . A better control of field fungi at 92% R.H. and of storage fungi at 85% R.H. was also observed, indicating that the efectiveness of the treatment may depend on conditions being more favorable to the development of a given group of fungi. This hypothesis may explain the minimun phosphine effect on non-growing fungi observed by Raghunatan *et al.*. (13) and Sinha *et al.*. (14).

For the two moisture contents (16% and 19.1%), the 1.0mg PH_3/L concentration allowed complete control of *Aspergillus* spp and *Penicillium* spp after the 7 days exposure period, while the 1.5mg PH_3/L concentration proved to be efficient after both periods (7, 14 days), confirming the previously mentioned coments by Castro *et al.*. (4).

Further studies using PH_3 for longer periods of exposition may clarify better the behaviour of storage fungi submitted to phosphine. There is a need for applied studies that should determine the most effective phosphine concentration acting at different moisture levels.

CONCLUSIONS

Under the experimental conditions used, the data obtained indicate that:

- The utilization of the fumigant phosphine for 7 and 14 days at the 1.0 and 1.5mg/L concentrations may be effective for the control of field and storage fungi in paddy rice grains with moisture contents at equilibrium with relative humidities of 85% and 92%.
- The 0.5mg/L phosphine concentration might have controlled the *Eurotium* spp development in paddy rice with moisture contents in equilibrium with relative humidities of 85% and 92%, after exposure for 7 and 14 days.
- The 1.0 and 1.5mg/L phosphine concentrations might have avoided the development of storage fungi in paddy rice with moisture contents at equilibrium with relative humidities of 85% and 92% after 14 days of exposure to the fumigant.
- The best control of field and storage fungi was observed in paddy rice submitted to relative humidities of 92% and of 85%, respectively
- The 1.0mg/L phosphine concentration was sufficient for the complete control of *Aspergillus* and *Penicillium* spp for a period up to 7 days, in paddy rice kept at 85% and 92% relative humidities.

ACKNOWLEDGEMENTS

The authors wish to thank Dra. Zofia K. Lawrence from the International Mycological Institute for her assistance on the fungi identification, Dra. Lúcia H.S. Mello de Castro from the Instituto Agronômico de Campinas for the raw material donation and to Mr. Marcio Costa from ITAL for their colaboration during the experiment.

RESUMO

Utilização de fosfina fumigante no controle de fungos naturalmente presentes em arroz em casca armazenado

Arroz em casca, recém-colhido, com atividades de água iniciais de 0,84 e 0,90 (equivalentes a 16,0% e 19,1% de teor de umidade em base úmida, respectivamente), foi exposto a 0,0; 0,5; 1,0 e 1,5 mg/L de fosfina (PH_3) por 7 e 14 dias, a 28°C. As concentrações do fumigante foram determinadas após cada período de exposição. A flora fúngica foi verificada antes e após todos os tratamentos utilizando-se o método do plaqueamento direto dos grãos sobre dois meios de cultura. Os resultados mostraram um decréscimo de grãos infectados com o aumento da concentração do fumigante. Em todos os tratamentos observou-se um melhor controle dos fungos de campo na U.R. de 92% e dos fungos de armazenamento na U.R. de 85%. A concentração de 0,5 mg/L de fosfina, provavelmente, foi suficiente para controlar o desenvolvimento de *Eurotium* spp em ambas umidades relativas.

Existe a possibilidade de que as concentrações de 1,0 e 1,5mg/L tenham sido eficientes no controle de fungos de armazenamento, nos dois períodos de exposição e nas duas umidades relativas estudadas. A menor percentagem de infecção dos grãos por fungos foi obtida na concentração de 1,5 mg/L, na U.R. mais elevada. O período de proteção oferecido pelo fumigante foi relacionado com a concentração aplicada.

Palavras-chave: arroz-armazenamento, fosfina, controle de fungos

REFERENCES

1. American Association of Cereal Chemists. *Approved Method of the American Association of Cereal Chemists*. St. Paul, 1976.
2. Annis, P.C. Sealed storage of bag stacks: status of the Ledinology. In: *Proceedings*. Fumigation and Controlled Atmosphere Storage of Grain, Singapore. 1989. p. 203-210.
3. Borsa, J.; Chelack, W.S.; Maerquardt, R.R.; Frohlich, A.A.; Charoe, N., Saovapong. Effects of different methods of desinfestation on mycotoxin production in grain: model studies with fumigation and irradiation in barley and rice. In: International Symposium on Stored-Grain Ecosystems, Winnipeg, Canada. *Resumos*, 60-62, 1992.

4. Castro, M.F.P.P.M.; Pacheco, I.A.; Taniwaki, M.H. Effects of phosphine on aflatoxin production in peanuts stored with a high content. In: *Proceedings*. Methyl Bromide Technical Options Workshop, Washington, USA. 1992.
5. Food and Agriculture Organization of the United Nations. Recommended methods for the detection and measurement of resistance of agricultural pests to pesticides. Tentative method for adults of some major pest species of stored cereals, with methyl bromide and phosphine. *FAO Plant Protection Bulletin*, Roma, 23(1):12-24, 1975 (FAO Method nº.16).
6. Harris, A.H. A conductimetric method for determining the concentrations of phosphine during fumigation. In: *Proceedings*. Gasca Seminar on Fumigation Technology in Developing Countries, Slough. London, T.D.R.I., 1986. p. 56-65.
7. Hocking, A.D.; Banks, H.J. Effects of phosphine fumigation on survival and growth of storage fungi in wheat. *J. Stored Prod. Res.*, 41(1):232, 1991.
8. Hocking, A.D.; Banks, H.J. Effects of phosphine fumigation on the development of storage mycoflora in paddy rice. In: *Proceedings*. International Working Conference on Stored-Product Protection, 5, Bordeaux, França.. 1991. p. 823-31.
9. Hocking, A.D.; Pitt, J.I. Dichloran-glicerol medium for enumeration of xerophilic fungi from low moisture foods. *Microbiol.* 39:498-492, 1980.
10. King, A.D.; Hocking, A.D.; Pitt J.I. Dichloran-rose bengal medium for enumeration and isolation of moulds from foods. *Appl. Environ. Microbiol.* 37:959-964, 1979.
11. Pelhate, J. Inventaire de la mycoflore des blés de conservation. *Bull. Trimest. Soc. Mycol. Fr.* 84:147-147, 1968.
12. Pitt, J.I.; Hocking, A.D.; Gleen, D.R. An improved medium for the detection of *Aspergillus flavus* and *Aspergillus parasiticus*. *J. Appl. Bacteriol.*, 54:104-114, 1983.
13. Raghunatan, A.N.; Majunider, S.K. Control of internal fungi of sorghum by fumigation. *J. Stored Prod. Res.*, 5:389-392, 1969.
14. Sinha, R.N.; Berek, D.; Wallace, H.A.H. Effect of phosphine on mites, insects and microorganisms. *J. Econ. Entomol.* 60:125-132, 1967.
15. Winston, P.W.; Bates, D.H. Satur2ated solutions for the control of the humidity in biological research. *Ecology*, 41(1):232, 1960.

Rev. Microbiol., São Paulo, **26**(3): 236-238, 1995

DECREASED SUSCEPTIBILITY TO ANTIBIOTICS AMONG *NEISSERIA GONORRHOEAE* ISOLATES IN FLORIANÓPOLIS (SC) - BRAZIL

Artur Smânia Junior*
Elza de Fátima A. Smânia*
Márcio Luiz Gil*

SHORT COMMUNICATION

ABSTRACT

Antibiotic susceptibility testing was performed on clinical isolates of *Neisseria gonorrhoeae*. The results obtained were as follows: 14.63% isolates were resistant to ampicillin, 45% to ceftriaxone, 16.67% to erythromycin, 29.27% to penicillin, 78.57% to tetracycline, and none to cefoxitin. Four strains were ß-lactamase producers, corresponding to 9.52% of penicillinase-producing *N. gonorrhoeae* in the population studied.

KEY WORDS: *Neisseria gonorrhoeae*, Antimicrobial Agents, ß-Lactamase.

Strains of *Neisseria gonorrhoeae* with reduced susceptibility to penicillin and other antimicrobial agents have increased markedly in recent years (2,15). Moreover, since the 1970s, penicillinase-producing *N. gonorrhoeae* (PPNG) have been reported at several locations (1,2,3,4,5,19). The situation applies to Brazil, where the first PPNG occurrence was documented in 1981 (12). Currently, strains have been reported from various Brazilian geographical areas (7,11,13,18). Thus, the present study was undertaken to reassess the resistance to antibiotics among *N. gonorrhoeae* isolates in Florianópolis, Brazil.

Specimens from suspected gonorrhoeae patients were obtained from public and private laboratories, throughout the 1991 to 1992 period. Initial isolations were made on Thayer-Martin medium. The cultures were incubated at 36°C under carbon dioxide (5-10%) atmosphere for 24-48 hours. Isolates were identified as *N. gonorrhoeae* if they were oxidase positive Gram-negative cocci and produced acid from glucose but not from maltose, sucrose or lactose. The strains were tested by the disk diffusion method, maintained in a semi-solid medium and stored at room temperature (17) for further studies.

The disk diffusion tests were performed on chocolate agar prepared with GC agar base (Difco) and 1% VX (Laborclin) as a supplement (9,18). The antimicrobial agents tested and the interpretative criteria used are shown in TABLE 1 (8,9,10,16). ß-lactamase production was tested by the acidometric method (18,20).

The results obtained with the diffusion test are shown in TABLE 2. ß-Lactamase was produced by 4 out of 42 (9.52%) *N. gonorrhoeae* isolates. Of these 4, only 2 were susceptible to cefoxitin and ceftriaxone, and one was susceptible to cefoxitin. The remainder strain was not susceptible to any of the drugs tested, but demonstrated intermediate

* Departamento de Microbiologia e Parasitologia - Centro de Ciências Biológicas - Universidade Federal de Santa Catarina - C. Postal 476 - 88040-900 - Florianópolis (SC) - Brasil.

TABLE 1. Interpretive criteria for antimicrobial susceptibility testing

Antibiotic (disk content)		Zone diameter (mm) Susceptible	Intermediate	Resistant
Ampicillin	(10µg)	≥40	39-22	≤21
Ceftriaxone	(30µg)	≥35	NA	≤34
Cefoxitin	(30µg)	≥28	27-24	≤23
Erythromycin	(15µg)	≥32	31-22	≤21
Penicillin G	(10U)	≥47	46-27	≤26
Tetracycline	(30µg)	≥38	37-31	≤30

NA, not applicable.

TABLE 2. Susceptibility to antibiotics among *Neisseria gonorrhoeae* isolates in Florianópolis (SC), Brazil.

Antibiotic	Susceptible Nº(%)	Intermediate Nº(%)	Resistant Nº(%)	Nº of isolates tested
Ampicillin	1(2.44)	34(82.93)	6(14.63)	41
Ceftriaxone	22(55.00)	NA	18(45.00)	40
Cefoxitin	25(59.52)	17(40.48)	0(0.00)	42
Erythromycin	3(7.14)	32(76.19)	7(16.67)	42
Penicillin G	0(0.00)	29(70.73)	12(29.27)	41
Tetracycline	2(4.76)	7(16.67)	33(78.57)	42

NA, not applicable.

susceptibility to cefoxitin.

Comparing the gonococcal susceptibility to antimicrobial agents in the present study with the data from an earlier report (18), a number of changes in antibiotic susceptibility patterns can be observed. While the prevalence of ß-lactamase-negative penicillin resistant strains appears to have declined from 54.32% (reported in 1989-1990) to 29.27% in the present study, the rate of ß-lactamase production increased from 3.70% to 9.52%. In addition, the rate of resistance to tetracycline increased from 69.14% (reported in 1989-1990) to 78.57%. In the first study, the expanded- and broad-spectrum cephalosporins were not used in the susceptibility test. However, none of the currently-tested isolates was found to be resistant to cefoxitin. This drug, together with ceftriaxone, was associated with the highest rate of susceptible strains. The data is in accordance with that reported by other authors (4,5,6). Various countries, including the United States (3,6), currently use cefoxitin and ceftriaxone as the drugs of choice for the treatment of gonorrhea. In contrast, in Brazil, penicillin G, ampicillin, and tetracycline are still recommended as first-line antibiotics for the treatment of gonorrhoeae (14).

Our data, and those previously reported, suggest that antibiotic resistance, including the penicillinase-producers, remains uncommon among gonococcal isolates in Florianópolis. It is clear that there is a need for further monitoring of antibiotic susceptibility and dissemination of the obtained information through medical and public health media.

ACKNOWLEDGMENTS

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Universidade Federal de Santa Catarina.

RESUMO

Aumento da resistência a agentes antimicrobianos de amostras de *Neisseria gonorrhoeae* isoladas em Florianópolis (SC) - Brasil

Foi avaliada a sensibilidade de amostras de *Neisseria gonorrhoeae* contra 6 agentes antimicrobianos. Observou-se que 14.63% dos isolados foram resistentes à ampicilina, 45% à ceftriaxona, 16.67% à eritromicina, 29.27% à penicilina, 78.57% à tetraciclina e nenhum à cefoxitina. Quatro amostras foram produtoras de ß-lactamase, representando uma ocorrência de 9.52% de *N. gonorrhoeae* produtora de penicilinase na população estudada.

Palavras-chave: *Neisseria gonorrhoeae*, agentes antimicrobianos, ß-lactamase.

REFERENCES

1. Brett, M.S.Y.; Davies, H.G.D.; Blokley, J.R.; Heffernan, H.M. - Antibiotic susceptibilities, serotypes and auxotypes of *Neisseria gonorrhoeae* isolated in New Zeland. *Genitourin. Med.*, 68: 321-324, 1992.
2. Centers for disease control - Follow-up on antibiotic resistant *Neisseria gonorrhoeae*. *Morbid. Mortal. Weekly Rep.*, 26: 29, 1977.

3. Centers for disease control - Antibiotic-resistant strains of *Neisseria gonorrhoeae*. *Morbid. Mortal. Weekly Rep.*, 36: 1S-18S, 1987.
4. Clendennen, T.E.; Echeverria, P.; Saengeur, S.; Kees, E.S.; Boslego, J.W.; Wignall, F.S. - Antibiotic susceptibility survey of *Neisseria gonorrhoeae* in Thailand. *Antimicrob. Agents Chemother.*, 36: 1682-1687, 1992.
5. Clendennen III, T.E.; Hames, C.S.; Kees, E.S.; Price, F.C.; Rueppel, W.J.; Andrada, A.B.; Espinosa, G.E.; Kabrerra, G.; Wignall, F.S. - "In vitro" susceptibilities of *Neisseria gonorrhoeae* isolates in the Philippines. *Antimicrob. Agents Chemother.*, 36: 277-282, 1992.
6. Fekete, T.; Woodwell, J.; Cundy, K.R. - Susceptibility of *Neisseria gonorrhoeae* to cefpodoxime: determination of MICs and disk diffusion zone diameters. *Antimicrob. Agents Chemother.*, 35: 497-499, 1991.
7. Franchini, M. - *Prevalência de Neisseria gonorrhoeae produtora de penicilinase no Distrito Federal*. I Encontro Nacional sobre Doenças Sexualmente Transmissíveis e III Simpósio sobre Doenças Sexualmente Transmitidas no Brasil Central, Brasília, DF, 1984.
8. Jones, R.N.; Fuchs, P.C.; Washington II, J.A.; Gavan, T.L.; Murray, P.R.; Gerlach, E.H.; Thornsberry, C. - Interpretive criteria, quality control guidelines, and drug stability studies for susceptibility testing of cefotaxime, cefoxitin, ceftazidime, and cefuroxime against *Neisseria gonorrhoeae*. *Diagn. Microbiol. Infect. Dis.*, 13: 499-507, 1990.
9. Jones, R.N.; Gavan, T.L.; Thornsberry, C.; Fuchs, P.C.; Gerlach, E.G.; Knapp, J.S.; Murray, P.; Washington II, J.A. - Standardization of disk diffusion and agar dilution susceptibility tests for *Neisseria gonorrhoeae*: Interpretive criteria and quality control guidelines for ceftriaxone, penicillin, spectinomycin, and tetracycline. *J. Clin. Microbiol.*, 27: 2758-2766, 1989.
10. Jones, R.N.; Gerlach, E.H.; Koontz, F.P.; Murray, P.R.; Pfaller, M.A.; Washington, J.A.; Erwin, M.E.; Knapp, C.C. - Interpretive criteria and quality control guidelines for *Neisseria gonorrhoeae* susceptibility test standardization for cefotetan. *J. Clin. Microbio.*, 29: 363-366, 1991.
11. Lombardi, C.; Siqueira, L.F.G.; Santos Jr., M.F.Q.; Francisco, W.; Belda, W. - *Neisseria gonorrhoeae* produtora de penicilinase: primeira cepa isolada em São Paulo, SP (Brasil). *Rev. Saúde Publ. São Paulo*, 19: 374-376, 1985.
12. Magalhães, M. - Uretrite causada por *Neisseria gonorrhoeae* produtora de penicilinase: relato de um caso. *Rev. Bras. Pat. Clin.*, 20: 116-118, 1984.
13. Magalhães, M. - *Neisseria gonorrhoeae* produtora de penicilinase no Recife, Brasil. *Rev. Microbiol.*, 18: 229-234, 1987.
14. Ministério da Saúde - Manual de Vigilância Epidemiológica das Doenças Sexualmente Transmissíveis - Aspectos Clínicos, 1993.
15. Morse, S.A.; Johnson, S.R. - Antimicrobial resistance among sexually transmitted pathogens. *Am. Soc. Microbiol. News*, 53: 201, 1987.
16. Ringertz, S.; Rylander, M.; Kronvall, G. Disk diffusion method for susceptibility testing of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.*, 29: 1604-1609, 1991.
17. Smânia, A.Jr.; Gil; M.L.; Smânia, E.F.A. - Um novo meio para a conservação de *Neisseria*. *Rev. Microbiol.*, 21: 366-368, 1990.
18. Smânia, A.Jr.; Gil, M.L.; Smânia, E.F.A.; Zoccoli, C.M.; Michels, D.; Lopes, R. - Resistência a agentes antimicrobianos de amostras de *Neisseria gonorrhoeae* isoladas em Florianópolis (SC). *Rev. Microbiol.*, 22: 308-312, 1991.
19. Tzelepi, E.; Fragouli, E.; Athanassopoulou, V.; Tzanakaki, G.; Tseliou, P. - *Neisseria gonorrhoeae* in Athens, Greece. *Sex. Transm. Dis.*, 18: 238-244, 1991.
20. Young, H. - Identification and penicillinase testing of *Neisseria gonorrhoeae* from primary isolation cultures on modified New York city medium. *J. Clin. Microbio.*, 7: 247-250, 1978.

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Guidelines to authors

Scope of the Journal

Revista de Microbiologia (Journal of the Brazilian Society for Microbiology), published by the Brazilian Society for Microbiology, is intended for publication of original research papers, research notes and, occasionally, reviews, covering all aspects of Microbiology.

Submitting manuscripts

Submission of a manuscript to Revista de Microbiologia (Journal of the Brazilian Society for Microbiology) is understood to imply that it has not previously been published (except in an abstract form) and that it is not being considered for publication elsewhere.
All manuscripts should be typewritten in English and submitted in triplicate to the most adequate Section Editor (names and addresses are listed in the front part of this issue).

Publication of a manuscript

Manuscripts are accepted for publication only after they are critically reviewed. Papers are reviewed by referees indicated by the Section Editor to whom the manuscript was submitted. After review, the manuscript will be returned to the nominated author for revision according to suggestions made by the reviewers. The author should return the reviewed manuscript to the Section Editor.

The author is notified when a manuscript is received and also when it is accepted or rejected for publication.

On acceptance of the paper, the nominated author will be requested to send the text on a computer diskette. Galley proofs will be sent to the author for correction. They should be checked carefully and handled promptly (5 days) according to instructions which are attached.

Membership in Brazilian Society for Microbiology is not a prerequisite for acceptance of a manuscript for publication. Nonmembers scientists from Brazil and other countries are invited to submit papers for consideration for publication.

Submission of a manuscript implies that <u>all</u> authors and their institutions have agreed to its publication.

Revista de Microbiologia assumes no responsability for errors made by the authors. Furthermore, Revista de Microbiologia assumes no responsability for conclusions reached by the authors.

Types of papers

The following types of papers are acceptable for publication in Revista de Microbiologia (Journal of the Brazilian Society for Microbiology):

Research paper: the research paper reports results of original research which have not been published elsewhere. It consists of 12 to 15 double-space typewritten or computer-written pages plus appropriate references, Tables and Figures. A summary with title (Resumo) and three to five key-words (palavras-chave) in Portuguese must also be included.

Short Communication: a Short Communication is a concise account of new and significant findings. It should be written according to the guidelines given for research papers (see below) but without the heading divisions. It's abstract and resumo (in Portuguese) should not exceed 50 words. Figures and Tables should be restricted to a maximum of two Figures or two Tables, or one Table and one Figure. The designation "short communication" will appear above the title of this type of paper. the author should specify that his manuscript is a short communication so that it can be properly evaluated during the review process.

Mini-review: Review articles should deal with microbiological subjects of broad interest. Specialists will be called upon to write them. In addition to an abstract in English and in Portuguese (resumo), they may contain a list of contents.

Preparation of Manuscripts

General

1 - All manuscripts should be typed double-spaced with wide margins and the pages should be numbered sequentially. Research papers should be restricted to 15 printed pages, including Figures and Tables. Short Communications should be restricted to 6 printed pages.
2 - All manuscripts should be submitted written in English. The Editor recommends that a manuscript should be read critically by someone fluent in English before it is submitted. Manuscripts in poor English will not be accepted.
3 - The paper should be organized in topics, as described in the next paragraph. The name of the topics should be typed in capital letters (e.g. ABSTRACT, INTRODUCTION, etc.).
4 - Abbreviations of therms and symbols should follow the recommendations of the IUPAC-IUB Commission and the Metric System is to be used throughout.
5 - As a rule, the references in the text should be cited by their numbers. Exceptionally, when authors are mentioned in the text, the mention should be done according to the following examples: Bergdoll (number) reported that..., Bailey and Cox (number) observed that..., or Smith *et al.* (number) mentioned that... Do not use capital letters.
6 - Authors of accepted papers will be requested to send a 3 1/2" diskette containing the text prepared in a P.C. based word processor.

Organization

Title page: A separate page should be used to give the title of the paper, complete name (including first name and middle initial) and affiliation of each author. An asterisk should be placed after the name of the author to whom correspondence about the paper should be sent. The telephone and fax numbers of this author should be given on the bottom of the page. No text of the manuscript should appear on the title page.
The title should be as brief as possible, contain no abbreviations and be truly indicative of the subject of the paper. Expressions such as "Effects of", "Influence of", "Studies on", etc., should be avoided. Care should be exercised in preparing the title since it is used in literature retrieval systems.

ABSTRACT: The abstract should be typed in a separate page and should not exceed 250 words. It should summarize the basic contents of the paper. The abstract should be meaningful without having to read the remainder of the paper. An abstract should not contain references, tables or unusual abbreviations. Abstracts are reprinted by abstracting journals and hence will be read by persons who do not have access to the entire paper. Hence the abstract must be prepared with great care. Three to five key words should also be included.

RESUMO: *RESUMO* is the abstract written in Portuguese. Its preparation should follow the same recommendations for the abstract in English. The *resumo* should also contain a title in Portuguese. The rules for the title in Portuguese are the same for the title in English (see above). Three to five *palavras-chave* (key words) have also

to be included. The *RESUMO* and the title in Portuguese should also be typed in a separate page.

INTRODUCTION: The introduction should begin on a new page and provide the reader with sufficient information so that results reported in the paper can be properly evaluated without refering to the literature. However, the introduction should not be an extensive review of the literature. The introduction should also give the rationale for and objectives of the study that is being reported.

MATERIALS AND METHODS: This section should provide enough information for other investigators to repeat the work. Repetition of details of procedures which have already been published elsewhere should be avoided. If a published method is modified, such modification(s) must be described in the paper. Sources of reagents, culture media and equipment (company, city, state, country) should be mentioned in the text. Names that are registered trade marks should be so indicated. Subheading often make this section easier to read and understand.

RESULTS: This section should, by means of text, tables and/or figures, give the results of the experiments. If a *DISCUSSION* section is to be included, avoid extensive interpretation of results but do so in the *DISCUSSION* section. If *Results* and *Discussion* are combined. then results should be discussed where, in the text, it is most appropriate. Tables should be numbered independently of the figures using Arabic numerals. All tables and figures must be mentioned in the text. The approximate location of tables and figures in the text should be indicated.

DISCUSSION: The discussion should provide an interpretation of the results in relation to known information.

ACKNOWLEDGMENTS: This section is optional and follows the *DISCUSSION*. It aknowledges financial and personal assistance.

REFERENCES: Arrange the references in alphabetical order, by last name of the author. All authors must be cited. Number the references consecutively. Cite each reference in the text by its number. Journal names should be abbreviated according to the style of *Biological Abstracts* or *Chemical Abstracts*. All references given in the list should be cited in the text and all references mentioned in the text must be included in the list. List references according to the style shown in the following examples.

a. Paper in a journal
Campos, L.C.; Whittam, T.S.; Gomes, T. A.T.; Andrade, J.R.C.; Trabulsi, L.R. *Escherichia coli* serogroup O111 includes several clones of diarrheagenic strains with different virulence properties. *Infect. Immun.*, 62: 3282-3288, 1994.

b. Paper or chapter in a book
Nelson, E.B. Current limits to biological control of fungal phytopathogens. *In*: Arora, D.K.; Rai, B.; Mukerji, K.G.; Knudsen, G. (eds). *Handbook of applied mycology; soils and plants*. Marcel Dekker, New York, 1991, p.327-355.

c. Book by author(s)
Salyers, A.A.; Whitt, D.D. *Bacterial pathogenesis. A molecular approach*. ASM, Washington, 1994, 418p.

d . Patent
Hussong, R.V.; Marth, E.H.; Vakaleris, D.G. Manufacture of cottage cheese. *U.S. Pat. 3,117,870*. Jan. 14, 1964.

e. Thesis
Calzada, C.T. Campylobacter jejuni *e* Campylobacter coli - *caracterização em sorogrupos e biotipos das cepas isoladas no município de São Paulo, no período de 1983-1989*. São Paulo, 1991, 131p. (Ph.D. Thesis. Instituto de Ciências Biomédicas. USP).

f. Publication with no identifiable author or editor
Anonymous. The economy of by-products. *Álcool Alcoolquim.*, 2;33-40, 1985.

g. Communications in events (Symposia, conferences, etc)
Simão, G.S.; Silva, J.; Toledo, A.S.; Gontijo Filho, P.P. *Micobactérias não tuberculosas isoladas de pacientes com a síndrome de imunodeficiência adquirida.* XVII Congresso Brasileiro de Microbiologia, Santos, 1993, p.41.

REFERENCES citing "personal communication" or "unpublished data" are discouraged, although it is recognized that sometimes they need to be used. In these cases, they should be cited in the text and not in the list of references. References consisting of papers that are "accepted for publication" or "in press" are acceptable. However, references of papers that are "submitted" or "in preparation" are not acceptable.

TABLES

Tables should not be included in the text. Each TABLE must be typed in a separate page and numbered sequentially with an Arabic number. The title of a TABLE should be placed in the top of it and should be brief but fully descriptive of the information in the TABLE. Headings and subheadings should be concise with columns and rows of data carefully centered below them.

FIGURES

Arabic numbers should be used for numbering the Figures. Data in Tables should not be repeated in FIGURES. The legend of the FIGURES should be placed in the bottom of them.

Photographs and line drawings

Only those photographs which are strictly necessary for the understanding of the paper should be submitted. Photoprints must be of sufficient quality to ensure good reproduction. They should be numbered on the back and identified with the nominated author's name. Legends of line drawings and photographs should not exceed the printing area. All elements in the drawing should be prepared to withstand reductions. Drawings and line figures should be drawn in black ink on tracing paper and should be prepared as indicated for the photographs. Colored illustrations are not accepted.

Reprints

Fifteen reprints of each paper will be mailed to the nominated author, free of charge. Additional reprints will be billed at cost price if requested upon returning the corrected galley proofs.

ERRATUM

Effects of sodium dodecyl sulfate on the growth of *Candida lipolytica*.
Nascimento, A.E.1; Campos-Takaki, G.M.2*
Vol. 26(2):140-143, 1995.

1. Laboratório de Imunopatologia Keizo Asami
2. Departamento de Antibióticos, Universidade Federal de Pernambuco - Campus Universitário Cidade Universitária, Recife - PE - Brazil - CEP 50670-901 - FAX (81) 271-8485
* To whom correspondence should be sent.