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EFFECT OF PROPIONIC ACID ON FUNGAL GROWTH AND AFLATOXIN PRODUCTION IN MOIST INSHELL GROUNDNUTS

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ABSTRACT

The effect of propionic acid (as ammonium salt) at 3.0 g/kg (**PA1**) and 5.0 g/kg (**PA2**) of inshell groundnuts (**IG**) was studied in laboratory conditions to verify its efficiency against fungal growth and aflatoxin (**AF**) production in rehydrated **IG** (16-18% moisture content). Fungal growth and aflatoxin contamination were evaluated on **IG** and their kernels before treatment (dry and rehydrated **IG**) and on days 7, 14, 21 and 28 of incubation. **PA1** treatment was ineffective in controlling fungal growth from day 14 on, when the average counts of treated samples were similar to those observed for control samples. Concerning the control of aflatoxin production, this treatment was effective till the 14th day of application. **PA2** treatment was efficient during the whole evaluation periods since the production of aflatoxins remained below 30 μg/kg of B_1+G_1 and the average counts of total and aflatoxigenic fungi were equivalent to those observed in dry **IG** samples (before rehydration).

Key words: aflatoxins, aflatoxigenic fungi, groundnuts, contamination, chemical control, propionic acid, post-harvest.

INTRODUCTION

Aflatoxins are substances produced by *Aspergillus flavus* Link and *A. parasiticus* Speare. B_1 aflatoxin is the major natural compound occurring within the aflatoxin group and is also the most carcinogenic, causing acute or chronic liver damage to most domestic and experimental animals and to humans (31). The aflatoxin-producing fungi are ubiquitous and can be easily isolated from many substrates. Among the economically important crops that may be invaded by these fungi and develop high levels of aflatoxins are the oilseed crops, especially groundnuts (11).

Among the environmental factors that favor invasion of pods and production of aflatoxin by *A. flavus* before and after harvest, the most important are relative humidity, and temperature (29,13). Other related factors include mechanical and biological damages and microbial competition (2,10), which depend on the nature of the substrate.

To avoid *A. flavus* development and subsequent aflatoxin production, groundnuts should reach a safe moisture content (pods < 10% and kernels < 8%) in 4 to 5 days after digging (12). However, these ideal values cannot be reached very often since natural drying under field conditions is dependent on the weather, which cannot be controlled.

Thus, the use of post-harvest chemical substances could be an option in controlling the development of aflatoxigenic fungi (8,16). Among the organic acids, propionic acid has been studied as a control agent against fungal development. *In vitro* doses that

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inhibited growth and aflatoxin production varied from 0.03% to 1.31% and were found to be pH dependent (1, 4, 19, 20, 33). Propionic acid was shown to control growth of aflatoxigenic fungi and aflatoxin production in maize kernels with high moisture content (28, 32). When windrowed groundnut pods were treated with propionic acid no aflatoxins were detected (17).

According to BASF (5) many studies show that the preservative effect of ammonium propionate is equivalent to that of propionic acid if the dosage is based on propionic acid concentration.

The present work was carried out to evaluate the effect of propionic acid (as ammonium propionate) on fungal growth (counts of total and potentially aflatoxigenic fungi) and aflatoxin production when sprayed on rehydrated inshell groundnuts.

MATERIALS AND METHODS

Preparation of samples. The experiment was conducted under laboratory conditions and a three randomized block design was applied with the following treatments: propionic acid at 3.0 g/kg (PA1), at 5.0 g/kg of inshell groundnuts (PA2) and Control.

The samples consisted of 500 g of hand-picked selected (HPS) mature inshell groundnuts of the "Tatu" cultivar (red skin, Valencia group) from the 1991 rainy season crop. The samples were rehydrated by adding water and were kept refrigerated at 4±2°C for 5 days till they reached approximately 16% moisture content. Subsequently, they were sprayed with 15 ml of propionic acid solutions, homogenized, put into polyethylene bags with cotton plugs and incubated at 30±2°C. Control samples were sprayed with the same volume of water.

The following evaluations were made before and after rehydration and at 7, 14, 21 and 28 days of incubation:

Moisture content. The moisture content was determined in two samples of 50 g by the oven method at 105±3°C during 24 hours (6).

Aflatoxin analysis. Aflatoxins were determined by the method of Pons Jr. *et al.* (25) with the following modifications: a) extraction with acetone+water (85+15); b) clean-up procedure with lead acetate solution without boiling (26); c) a the volume of chloroform for partitioning (3) was 2x25 ml.

The treatment was considered efficient when the aflatoxin content (B_1+G_1) remained under 30 µg/kg, which is the maximum limit allowed by Brazilian laws (7).

Mycological evaluation. The evaluation of total and aflatoxigenic fungi was made by serial dilution taking 100 g of inshell groundnuts and 50 g of their kernels, obtained aseptically after washing the pods with 0.5% NaHClO solution (24). Aliquots of 1mL were inoculated by the Pour Plate method, in duplicate, in Dichloran Rose Bengal Chloramphenicol (DRBC) and in *Aspergillus flavus parasiticus* Agar (AFPA) media. The DRBC plates were incubated at 30±1°C for 4-5 days when total fungi were counted (15), and the AFPA plates were incubated at 30±1°C during 42-48 hours when potentially aflatoxigenic fungal colonies were counted (23).

In parallel, monitoring fungi genera present in DRBC plates was conducted. The prevalent fungi were isolated and identified on Malt Extract Agar (MEA) and Czapeck Yeast Extract Agar (CYA) (24, 27).

RESULTS AND DISCUSSION

The average moisture content of samples after rehydration were adequate for fungal growth and aflatoxin production during the incubation period (Table 1).

Samples of inshell groundnuts taken before treatment did not present detectable levels of aflatoxins. Following rehydration and refrigeration, the pods showed an increase in the average count of total fungi compared with samples before rehydration, with prevalence of *Penicillium* spp. and yeasts.

A decrease in the average counts of aflatoxigenic fungi on pods and kernels was observed. Bacteria (rods) were detected on AFPA plates from dilutions 1:10 and 1:100.

There is evidence showing that bacteria are associated with internal tissues of the groundnut plant without being pathogenic (35) or interfering with the aflatoxigenic or fungal counts (23). However, a study of *Bacillus subtilis*, which is frequently found in groundnut pods (22), reported that inhibition of aflatoxigenic fungi and of aflatoxin production may occur depending on the bacterial strain examined (14).

In the present work, although bacteria were not identified, they could be the cause of the reduced fungal counts on AFPA plates. An additional factor could be the low refrigeration temperature (4±2°C), not ideal for the development of aflatoxigenic fungi.

Table 1. Average (*) moisture content (%) of inshell groundnuts during the experimental period

Period	PA1	PA2	Control
Before rehydration	7.8	7.8	7.8
After rehydration	17.3	17.2	17.3
After 7 days of incubation	17.7	17.8	18.0
After 14 days of incubation	17.4	17.1	17.1
After 21 days of incubation	15.7	15.3	17.1
After 28 days of incubation	12.6	14.5	16.5

(*) Average of 9 repetitions before and after rehydration and average of 6 repetitions during the incubation periods.
PA1 = Propionic Acid at 3.0 g/kg of inshell groundnuts
PA2 = Propionic Acid at 5.0 g/kg of inshell groundnuts

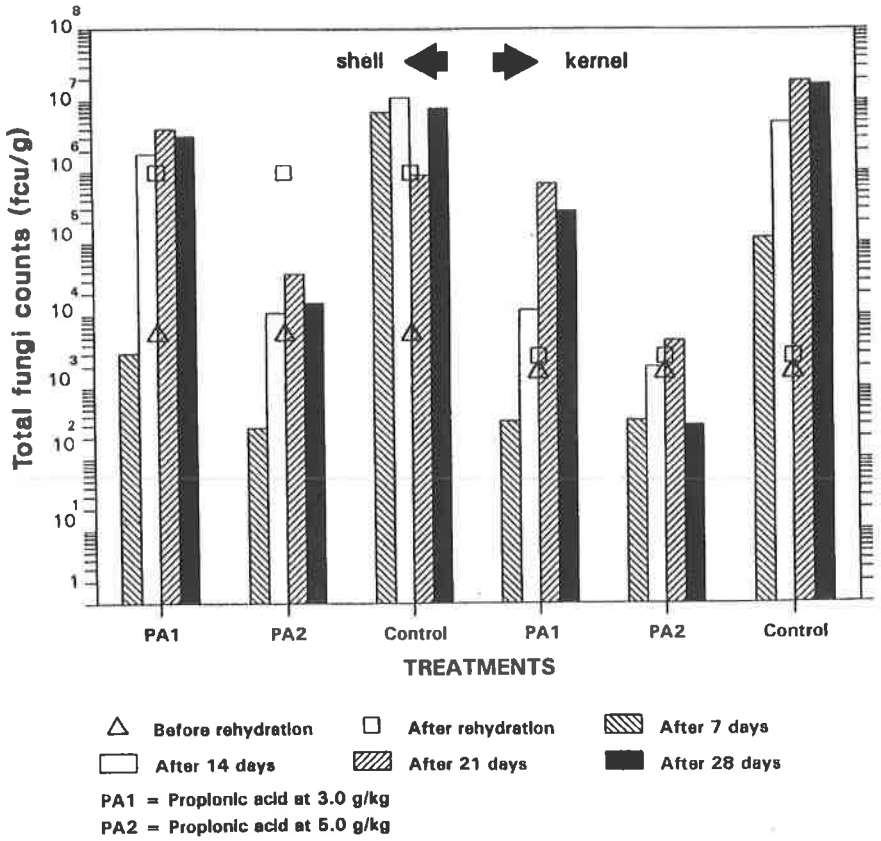

Figure 1. Average counts of total fungi on moist inshell groundnuts treated with propionic acid

Consequently, these average counts are not comparable with the values observed after treatments.

Total fungal count. The lowest average count of total fungi was observed with treatment **PA2** (pods and kernels) for any evaluation period (Fig. 1). On the 7th day of incubation, the average fungal counts for both **PA1** and **PA2** treatments (pods and kernels) were below the average values observed for groundnut before rehydration. This shows that, at the beginning, both doses of propionic acid were equally efficient. From 14th day of incubation onwards only treatment **PA2** maintained the lowest average counts, though a trend towards increasing values was detected. The average count for **PA1** was equivalent to those recorded for control samples (pods and kernels). It was therefore concluded that the **PA1** treatment was not efficient in controlling fungal contamination during

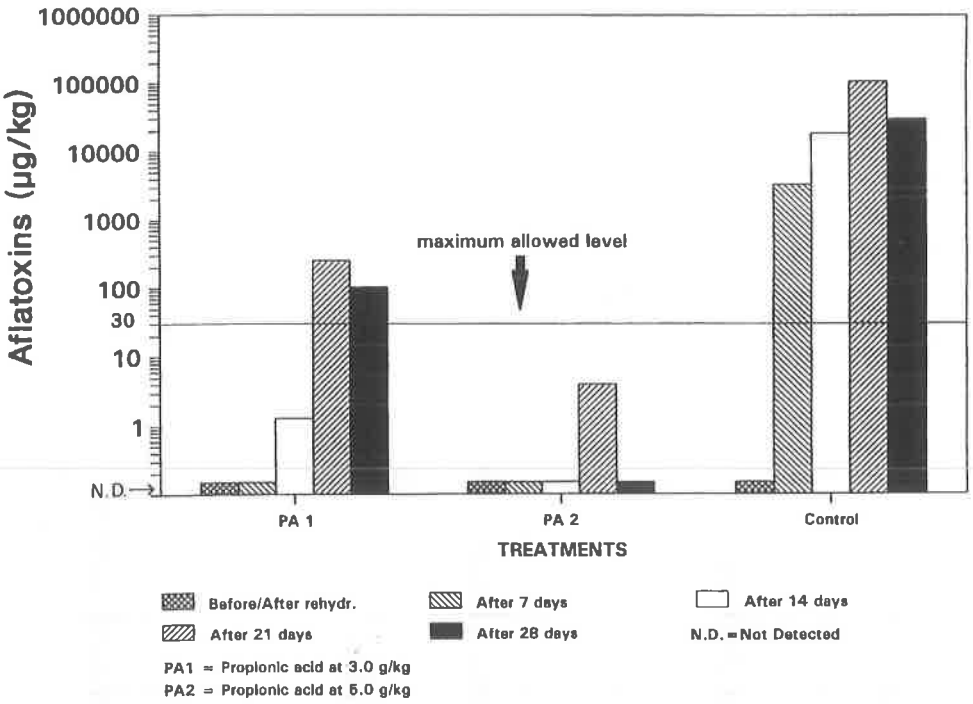

Figure 2. Mean aflatoxin contents in moist unshelled groundnuts treated with propionic acid

the whole incubation period. Treatment with propionic acid at the two doses tested showed a selective effect on the natural micoflora, when *Eurotium* sp. represented more than 50% of total fungal counts on DRBC plates (pods and their kernels). This effect was evident with treatment **PA1** and could have determined the lowest average counts of potentially aflatoxigenic fungi up to day 14 of incubation. It has been reported that subinhibitory doses together with inadequate distribution of chemicals could favor fungal growth on the treated material with an initial low level of contamination. A change in the sequence of fungi thus occurs, enabling the growth of species tolerant to treatment (32,16). The prevalence of species of the *A. glaucus* group has been observed on maize, sorghum and triticale stored with high moisture content after treatment with propionic acid (30,21). *In vitro* studies have demonstrated that *A. glaucus* strains are less susceptible to propionic acid than *A. flavus* and other *Aspergillus* species (18).

Potentially aflatoxigenic fungal counts and aflatoxin production. It was observed that, throughout the evaluation period, treatment **PA2** was able to keep the level of contamination with potentially aflatoxigenic fungi similar to that recorded for inshell groundnuts before rehydration. No aflatoxins were detected with treatment **PA2** except on day 21 of incubation, though the detected average level (4 µg/kg) was under the maximum limit allowed of 30 µg/kg (Fig. 2). With treatment **PA1**, on the other hand, the counts for aflatoxigenic fungi (pods and kernels) on days 21 and 28 of incubation increased above the average values observed before rehydration (Fig. 3). Aflatoxin production was detected at concentrations of 257 and 104 µg/kg on days 21 and 28 of incubation (Fig. 2). When no aflatoxins were detected, potentially aflatoxigenic fungi recovered from kernels were tested for their aflatoxin producing ability by the method of David *et al.* (9) and showed positive results.

The effectiveness of treatment **PA2** observed during the whole evaluation period might be explained by the volatility of this compound, which should facilitate it reaching the kernels yet might be a problem due to up to 70% acid loss during spraying (32).

The inhibitory action of propionic acid has been demonstrated in maize stored with high moisture content. A dose of 0.4% added directly to rehydrated maize controlled contamination for more than 35 weeks (30). When high moisture maize was heavily inoculated with *Aspergillus flavus/parasiticus* and treated with 1% propionic acid, growth and formation

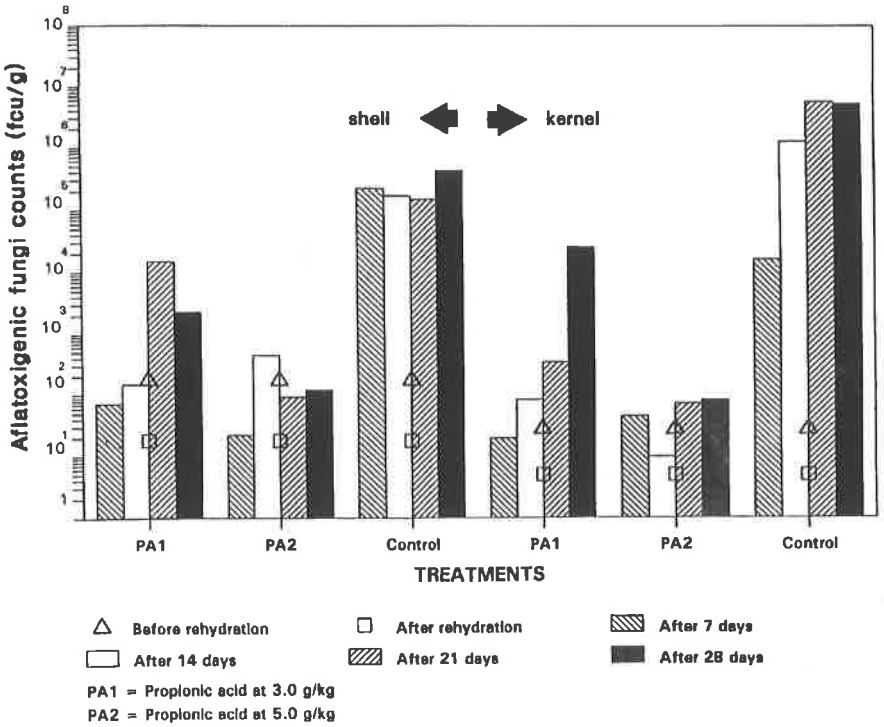

Figure 3. Average count of potentially aflatoxigenic fungi from moist inshell groundnuts treated with propionic acid

of aflatoxins were inhibited for about 19 weeks (34). Under natural conditions, when groundnut plants were harvested at a proper stage of maturity, inverted in windrows and their pods immediately sprayed with 5.0% propionic acid solution, *Aspergillus flavus* growth and aflatoxin production could be controlled on pod shells and kernels (17). Studies carried out *in vitro* demonstrated that the ability of propionic acid to inhibit fungal growth and aflatoxin production is dependent on its concentration. So germination and aflatoxin production can occur after enough acid has been metabolized by spores (1, 20; 28).

Future studies must consider the factors mentioned above when evaluating the usefulness of propionic acid under weather conditions adverse to rapid drying in the field. Other considerations include studies of the application system for groundnut pods and possible changes in the organoleptic properties of kernels.

CONCLUSIONS

Considering the conditions of this experiment, propionic acid (as ammonium salt) at 5.0 g/kg (0.5%) was able to control the growth of fungi, including aflatoxigenic species, as well as the production of aflatoxins. Therefore, propionic acid at this dose is a promising substance to be tested under field conditions for control of aflatoxin production in moist inshell groundnuts.

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RESUMO

Efeito do ácido propiônico sobre o crescimento fúngico e a produção de aflatoxinas no amendoim em casca úmido

O efeito do ácido propiônico (como sal de amônio) nas concentrações de 3,0 g/kg (AP1) e 5,0 g./kg (AP2) foi avaliado em laboratório para verificar sua eficiência sobre o crescimento fúngico e produção de aflatoxinas quando aplicado sobre amendoim em casca úmido (16 a 18% de umidade). As avaliações,

crescimento fúngico sobre as vagens e seus grãos e as análises de aflatoxinas, foram realizadas antes da incubação (amendoim em casca seco e reidratado) e aos 7, 14, 21 e 28 dias de incubação. O tratamento AP1 foi ineficiente no controle do crescimento fúngico a partir do 14º dia quando as contagens de fungo foram similares àquelas encontradas nas amostras controle, enquanto que, no que diz respeito ao controle da produção de aflatoxinas, este tratamento foi eficiente até o 14º dia. O tratamento AP2 foi eficiente durante todo o período de avaliação, no qual a produção de aflatoxinas manteve-se abaixo de 30 μg/kg (B_1 + G_1) e as médias de contagem total e de fungos potencialmente aflatoxigênicos foram equivalentes àquelas observadas nas amostras de amendoim em casca antes da reidratação.

Palavras-chave: aflatoxinas, ácido propiônico, fungos aflatoxigênicos, amendoim, contaminação, controle químico.

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COLONIZATION POTENTIAL OF BANANA LEAVES FOR GROWTH OF *PLEUROTUS* SPECIES

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ABSTRACT

A colonization test was carried out to evaluate the potential of dried banana leaves (**BL**) for growth of edible mushrooms. Banana leaves were used singly or mixed with corn cobs (**BLCC**)(70:30) and sugar cane bagasse (**BLSB**) (50:50). Wheat straw (**WS**) was used as control substrate. Three different species of the genus *Pleurotus* were tested, namely: *Pleurotus* sp. "Florida", *P. ostreatoroseus* and *P. sajor-caju*. The dried wastes were chopped into particles of about 0.5 cm in length, rehydrated to reach approximately 70% moisture content and sterilized in petri dishes at 120°C for two hours. The pH values were 7.0 for **BL** and **WS**, 6.3 for **BLSB** and 7.4 for **BLCC**, not requiring further adjustment. Experiments were performed in triplicate for each substrate/taxon combination. After sterilization, the substrates were inoculated with a colonized disk culture (1.0 cm) and incubated for 12 days at 25±1°C. Radial growth was measured every four days in parallel with a visual evaluation of mycelial vigor. All the substrates supported mycelial growth of the cultures tested. Considering the average radial growth, its lowest standard deviation and mycelial vigor, the best results were obtained with **BL** inoculated with *P. ostreatoroseus* or *P. sajor-caju* and with **BLCC** inoculated with *P.* sp. "Florida" or *P. ostreatoroseus*. For substrate **BLSB,** optimum growth was generally associated with lower mycelial vigor. The control substrate (**WS**) showed the smallest radial growth and highest mycelial vigor for all the cultures tested.

Key words: colonization, *Pleurotus*, edible mushroom, banana leaves.

INTRODUCTION

Pleurotus spp. are mushrooms found in nature as saprophytes and primary decomposers on dead wood. The ability to decompose lignin, cellulose and hemicellulose is related to the capacity of these higher fungi to secrete a large spectrum of hydrolytic and lignolitic enzymes, which allow them to colonize a wide variety of agricultural wastes without previous fermentation treatment (14). Biodegradation of these unmodified lignocellulosic residues by mushroom cultivation of edible species opens the possibility to convert them into highly nutritive and palatable foodstuffs.

Traditionally, edible *Pleurotus* species are cultivated on rice and wheat straw. However, new experimental agrowastes are being tested for commercial cultivation, like corn cobs mixed with wheat straw at a 70:30 ratio (11), sugarcane bagasse, banana leaves (1), banana pseudostems and banana

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trash (9). The best results using corn cobs are apparently obtained when these are mixed with other plant residues (8,13). A similar effect was observed with sugarcane bagasse (2,7). In Mexico, edible species of *Pleurotus* are gaining importance through the research work performed since 1982 at the National Research Institute of Biotic Resources (INIREB) (6). These mushrooms have been successfully cultivated for commercial purposes using a wide variety of agricultural wastes, including coffee pulp (Mexico) and wheat straw mixed with citronella bagasse (Guatemala) (6).

About 38 species of the genus *Pleurotus* have been described, though only 31 of them are edible and grow wild in Tropical America (6). At present, however, the cultivation of edible mushrooms is restricted to the genus *Agaricus* (11 countries) (6,16) and yet *Pleurotus* species show some advantages over *Agaricus* species since they are better adapted to tropical regions and do not require the use of greenhouses with an artificially created climate. They also have a shorter life cycle, which implies a higher turnover in the form of fruit bodies, and can grow on a wide range of unfermented plant wastes. Furthermore, the remains of their bioconverted substrates have many potential applications as upgraded forms of ruminant feed and garden manure, and may be used in the production of biogas, saccharolytic enzymes, etc. (10).

Research on low cost agricultural wastes for cultivation of *Pleurotus* species aims at reducing the cost of production and is also interesting from an ecological standpoint.

The first step for studying the potential of agrowastes is to know their colonization potential for growth of *Pleurotus* mycelia.

Banana leaves are found in great quantity in "Vale do Ribeira", state of São Paulo, one of the major banana producing areas of Brazil. Thus, the aim of the present work was to evaluate the potential of banana leaves, singly or mixed with either corn cobs or sugarcane bagasse, as substrates for colonization by three selected edible species of *Pleurotus*, namely: *P.* sp. "Florida", *P. sajor-caju* and *P. ostreatoroseus*. The first two taxa were chosen due to their optimum temperature (25-30°C) for colonization (spawn run) (9). *P. ostreatoroseus* was selected for being a native tropical taxon (2). Wheat straw was used as control substrate.

MATERIALS AND METHODS

Cultures. Pure cultures of *Pleurotus*, supplied by "Instituto de Botânica de São Paulo", included *Pleurotus* sp. "Florida" (001), *P. sajor-caju* (020) and *P. ostreatoroseus* (016). Cultures were maintained on agar slopes in PDA medium (Potato Dextrose Agar - DIFCO). The culture tubes were kept under refrigeration (5±2°C).

Inocula. Inocula were prepared from culture tubes for the colonization test. A fragment of mushroom mycelium was removed for growth on petri dishes containing PDA and prepared in duplicate for each taxon. Incubation was carried out at 25±1°C for 7 days.

Substrates. The substrates studied were: dried banana leaves (**BL**); **BL** mixed with sugarcane bagasse (**BLSB**), 50:50 on dry weight; **BL** mixed with corn cobs (**BLCC**), 70:30 on dry weight. Wheat straw (**WS**) was used as control.

The physical structure of the substrates was standardized by grinding in a blender so as to obtain particles of no more than 0.5 cm in length.

About 4 to 8 g of each substrate were placed in Petri dishes. Next, distilled water was added to give a final moisture content of about 70%. The quantity of water to be added was calculated on the basis of dry matter, determined by the oven method at 135° C for 2 hours (4). The Petri dishes with the identified substrates were sterilized at 121°C for 2 hours.

Three replicates were prepared with each substrate/taxon combination. Two replicates for each substrate were prepared to monitor moisture adjustment and for pH determination. The pH was measured using the watery extract of each substrate (12).

Colonization test. Substrates were inoculated with 1cm sterile agar discs colonized with the mycelium of each culture studied. The agar discs were placed in the center of the substrate's surface for uniformity of measurements. Incubation was carried out at 25±1°C for 12 days.

Evaluation of growth. Radial Growth: Radial growth was evaluated every four days, starting on the day of inoculation. The measurements were taken from the bottom side of the Petri dish and correspond to the average of two perpendicular diameters, drawn from a streak previously marked as origin.

The results for each substrate/taxon combination are presented as the mean of three replicates with respective standard deviation.

Mycelial vigor: The vigor (strength and energy) of mycelial development was visually analyzed using a subjective scale. Values of 1 to 5 were defined as follows: 1 (*) = low and sparse mycelium spreading across the Petri dish (faint mycelial growth); 2 (**) = relatively low and sparse mycelium spreading across the Petri dish; 3 (***) = fairly cottony and rather dense mycelium; 4 (****) = cottony and rather dense mycelium; and 5 (*****) = very cottony mycelium.

RESULTS AND DISCUSSION

Moisture content and pH of substrates after sterilization. Some physical and chemical features of a substrate are essential for the colonization period. Moisture content and pH are two factors that must be determined when new substrates are under study (9).

The moisture content of substrates is apparently more adequate when at around 70-75% and depends on the water-retaining capacity of the material (9); pH values between 5.0 and 7.0 and the presence of nutrients are also necessary for mushroom growth (10,15).

In the present work, the moisture obtained after adjustment was 69.25% for **BL**, 68.25% for **BLSB**, 70.25% for **BLCC** and 72.48% and for **WS**.

The pH values for the substrates are presented in Table 1. It is important to emphasize that substrates under natural conditions of cultivation are not sterilized. Therefore, at pH above 7.0, these can be easily contaminated by other organisms and may present an increase in bacterial populations. At pH values below 4 inhibition of mycelial growth occurs (10).

Colonization test. Mushroom production depends on rapid colonization (fast growth rate) and profuse mycelium formation. These features reflect the capacity of a substrate to be colonized and are directly related to the decomposition ability of *Pleurotus* species. A rapid colonization is favorable because it reduces the hazard of substrate contamination and also permits short incubation and fruitification periods (9).

Considering the results obtained (Table 1) it can be concluded that all the substrates evaluated support the mycelium growth of the three taxa studied.

For all the *Pleurotus* species tested, the greatest mycelium vigor and smallest radial growth were observed on substrate **WS**. This could be related to the physical structure of the straws that, after grinding, presented particles more uniform in size compared with the other substrates. The uniformity of particles may have contributed to a better availability of nutrients, resulting in greater mycelial vigor as well as more adequate moisture content.

In general, for all the taxa studied, the greatest radial growth was associated with a small mycelial vigor, particularly with substrate **BLSB**. This is probably due to physical and chemical characteristics

Table 1. Substrate colonization by edible *Pleurotus* species

SUBSTRATE	Colony diameter[a] *(cm)*								
	***P.* sp. "Florida"**			***P. ostreatoroseus***			**P. *sajor-caju***		
	4th day	**8th day**	**12th day**	**4th day**	**8th day**	**12th day**	**4th day**	**8th day**	**12th day**
BL (pH=7.0)	*[c] 3.50 (0.76)[d]	* 5.73 (0.87)	*** 6.93 (1.60)	** 2.63 (0.32)	** 5.66 (0.29)	*** 8.16 (0.57)	** 4.43 (0.07)	** 6.50 (0.00)	**** total[b] (0.00)
BLSB (pH=6.3)	** 3.76 (0.46)	** 6.90 (0.97)	** total (0.00)	* 3.93 (0.51)	* 7.10 (0.65)	** total (0.00)	* 4.86 (0.15)	* 7.30 (0.17)	** total (0.00)
BLCC (pH=7.4)	** 3.76 (0.21)	** 6.16 (0.57)	*** 8.16 (0.57)	** 3.80 (0.00)	** 6.33 (0.29)	*** 8.00 (0.86)	** 3.93 (0.12)	** 6.16 (0.57)	*** 8.16 (0.57)
WS (pH=7.0)	**** 2.83 (0.31)	**** 4.33 (0.82)	**** 5.63 (0.00)	*** 2.50 (0.00)	**** 3.50 (0.29)	**** 5.00 (0.88)	***** 3.26 (0.46)	***** 4.76 (0.40)	***** 6.16 (1.04)

[a] mean values obtained from three replicates
[b] Petri dishes with full mycelium growth = 9.0 cm of diameter.
[c] Mycelial vigor = * faint; ***** densely cottony; values within this range were defined as intermediate features.
[d] Standard deviation
Legend: **BL**=banana leaves; **BLSB**=BL+sugarcane bagasse(50:50, on dry matter); **BLCC**=BL+corn cobs(70:30, on dry matter); **WS**=wheat sraw

of the substrates. Considering the physical features of **BL** and its mixtures, a certain degree of heterogeneity was present with respect to particle diameter.

It has been reported that the particle size of the vegetal residue influences the decomposition ability of *Pleurotus* species, particularly when it is made of different tissues with different physical and chemical properties. Thus, when the structures of the cellular walls are made of less rigid components they are more easily degraded. This was observed with sugarcane bagasse (7).

Other considerations to explain the faint mycelial vigor observed for substrate **BLSB** relate to the chemical composition of sugarcane bagasse and its moisture content. The moisture content of this substrate was the lowest, 68.25%, and below the minimum value recommended. The presence of sucrose in the chemical composition of sugarcane bagasse is also of importance since *Pleurotus* species are not able to use this disaccharide for growth (9), although some authors have reported its utilization (1,3,5,7).

Considering the lowest standard deviation, the average radial growth and mycelial vigor, the best results were obtained with the combination of substrate **BL** with *P. ostreatoroseus* or *P. sajor-caju* and of substrate **BLCC** with *P. ostreatoroseus* or *P.* sp. "Florida". *P.* sp. "Florida", despite presenting a similar mycelial vigor on substrates **BL** and **BLCC** on the 12^{th} day of incubation, showed a smaller radial growth in the former with higher standard deviation.

It is hoped that the studied agrowastes will become competitive as substrates for the production of edible *Pleurotus* species. However, future studies will have to include an analysis of their chemical composition and evaluation of their potential for fruitification. Productivity measurements will also have to be taken into account to establish how economically feasible the commercial utilization of banana leaves is.

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RESUMO

Teste de colonização em palha de folha de bananeira por espécies de *Pleurotus*

Um teste de colonização por *Pleurotus* sp. "Florida", *P. ostreatoroseus* e *P. sajor-caju*, fungos comestíveis, foi realizado visando conhecer a potencialidade da palha de folha de bananeira (**PB**), resíduo lignocelulósico, como substrato para o cultivo desses fungos. Utilizou-se a **PB** e esta misturada ao bagaço de cana (**PBBC**) ou sabugo de milho (**PBSM**). A palha de trigo (**PT**) foi empregada como parâmetro de comparação por ser o resíduo tradicionalmente empregado. As palhas picadas, em partículas de aproximadamente 0,5 cm de comprimento, foram reumedecidas até alcançar aproximadamente 70% de umidade; em seguida foram esterilizadas, em placas de Petri, a 120°C por 2 horas. O pH dos substratos foi o seguinte: 7,0 no **PB** e **PT**, 6,3 no **PBBC** e, 7,4 no **PBSM**, não necessitando de ajuste. Após esterilização, os substratos foram inoculadas com discos de agar colonizado, permanecendo em estufa a 25±1°C por 12 dias. As avaliações realizadas foram: medidas de crescimento radial tomadas a cada 4 dias e vigor das colônias em crescimento de acordo com uma escala subjetiva. Para cada combinação taxon/substrato prepararam-se placas em triplicata. Todos os substratos estudados suportaram o crescimento do micélio das três culturas. Considerando-se as médias de crescimento e os menores desvios padrão destas e o vigor micelial, os melhores resultados para o substrato **PB** foram obtidos com *Pleurotus ostreatoroseus* e *P. sajor-caju*; para o **PBSM** com *P.* sp. "Florida" e *P. ostreatoroseus*. O substrato **PBBC** apresentou, para todas as culturas, a maior taxa de crescimento, que esteve relacionada, no geral, a um menor vigor micelial; para o substrato **PT**, o inverso foi verdadeiro: o menor crescimento radial relacionou-se ao maior vigor micelial.

Palavras-chave: colonização, cogumelo comestível, *Pleurotus*, folha de bananeira.

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CANDIDA ALBICANS : BIOTYPING BY SUSCEPTIBILITY TO ANTIMYCOTICS

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ABSTRACT

The "in vitro" antifungal activity of amphotericin B, ketoconazole, miconazole and flucytosine against 100 *Candida albicans* strains recovered from different clinical specimens was studied. Amphotericin B at a concentration of 1.0 μg/mL was the most effective agent, inhibiting 90.0% of all the strains tested. Ketoconazole at the concentration of 8.0 μg/mL was the least effective, inhibiting only 37.0% of the *C.albicans* isolates. Susceptibility or resistance to the 4 antifungal agents were respectively detected in 10.0% and 1.0% of the isolates. Based on susceptibility scores for amphotericin B, ketoconazole, miconazole and flucytosine, a digital system called "antifungaltyping" was developed which allowed the identification of 12 different types or patterns. Biotype 1211 (25%) was the most frequently isolated followed by biotype 1221 (20%). Strains isolated from a single patient at different times repeatedly showed type 1211 in skin lesions and type 2221 in bronchial washing; type 1211 was isolated from different specimens (sputum and oropharingeal swab) of the same patient on the same occasion, likewise type 1121 (bronchial washing and sputum).

Key words: *Candida albicans*, biotypes, amphotericin B, ketoconazole, miconazole, flucytosine.

INTRODUCTION

Opportunistic infections caused by yeasts of the genus *Candida* are becoming more frequent. *C.albicans* has been recovered from infections occurring in different groups of patients, specially those undergoing long term therapy or suffering from diseases that affect the immune system, like cancer, diabetes, severe burns of open wounds (10, 13). The antifungal agents amphotericin B, ketoconazole, miconazole and flucytosine are available for use in parenteral therapy of fungal infections (6, 9, 10, 17). Resistance to each of these agents has been clearly documented among clinically significant isolates of fungi (6, 17, 20). New drugs like fluconazole and itraconazole, among others, have been introduced in antifungal oral therapy (6, 9, 10).

Epidemiological studies for the characterization of pathogenic yeasts have been proposed in order to further classify these organisms into subgroups or biotypes (11, 13, 15).

Previous studies reporting strain identification and differentiation by serotyping (7), biotyping by a series of plate tests (12), the killer system (15) and morphotyping (14), besides several molecular epidemiological methods, have been performed (11, 13).

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The present investigation was carried out to evaluate the minimal inhibitory concentrations (MIC) of amphotericin B, ketoconazole, miconazole and flucytosine against 100 strains of *C.albicans*. Based on susceptibility scores, a digital system to differentiate types was developed.

MATERIALS AND METHODS

Organisms. A total of 100 *C.albicans* strains isolated from a variety of clinical specimens (stools, urine, sputum, blood culture, skin lesions, pharyngeal swab, etc.) from 93 patients was studied.

The isolates were cultured on Sabouraud's dextrose agar (SDA) plus 50 µg/mL cloramphenicol. *C.albicans* strains were identified by the germ tube test and chlamydospore production (17). The strains were maintained on SDA at room temperature. *S.cerevisiae* ATCC 9763 and *C.pseudotropicalis* "Carhston" were used as control strains.

Inoculum. The inoculum was prepared in sterile saline from 24-48h old cultures incubated at 30°C on SDA. The cells were counted in a hemocytometer and diluted to 1-2 x 10^6 cells/mL (19).

Susceptibility testing. The antifungal agents amphotericin B (Squibb), ketoconazole, miconazole (Johnson & Johnson) and flucytosine (Roche) were used. Stock solutions of amphotericin B, ketoconazole and miconazole were prepared in dimethyl sulfoxide; flucytosine solution was prepared in distilled water (19). The solutions were stored at -20°C and used within a week of their preparation.

Minimum inhibitory concentrations (MIC) were determined by the agar dilution method (19). A yeast nitrogen base (Difco) supplemented with 10.0% dextrose (YNBD) in 1.1% agar, pH 7.0, (3), was the test medium used.

Each antifungal agent was adjusted to a concentration of 2560 µg/mL in YNBD and immediately diluted by serial twofold dilutions in YNBD to concentrations ranging from 1280 µg/mL to 0.625 µg/mL. Final drug concentrations in the agar media ranged from 128 µg/mL to 0.062 µg/mL.

A Steers replicator was used to inoculate the strains on the Petri dishes. Drug-free plates were also inoculated. MIC values were determined 48h after incubation at 30°C and expressed as µg/mL. The MIC was defined as the lowest concentration of drug that completely inhibited the growth of the yeast. All assays were performed in duplicate.

Typing of *C.albicans* according to susceptibility to antifungal agents. *C.albicans* was typed according to the following criteria: strains with MIC ≤ 1.0 µg/mL for amphotericin B and ≤ 8.0 µg/mL for ketoconazole, miconazole and flucytosine were considered susceptible and ranked by the numeral 1; strains with higher MIC were considered resistant and ranked by the numeral 2. Coding, which was called "Antifungaltyping", consisted of 4 digits, each representing one of the antifungal agents in the order listed above. As an example, the 1221 "antifungaltype" denotes susceptibility to amphotericin B (MIC ≤ 1.0 µg/mL) and to flucytosine (MIC ≤ 8.0 µg/mL) and resistance to ketoconazole and miconazole (MIC > 8.0 µg/mL).

Discriminating power. The discriminating index-D (8) was used to verify the discriminatory power of the "antifungaltyping" technique.

RESULTS

The majority of the isolates (90.0%) were inhibited by 1 µg/mL amphotericin B. The ketoconazole MIC for 37% of the *C.albicans* clinical isolates was 8.0 µg/mL. Miconazole at 8.0 µg/mL inhibited 50.0% of the tested strains. Flucytosine MIC values of 8.0 µg/mL were verified for 78% of the *C.albicans* strains.

Table 1 summarizes the MIC in terms of range and geometric (G) mean MIC50 and MIC90 values.

On the basis of the MIC values it was possible to identify 12 "Antifungaltypes," with predominance of type 1211 in 25.0% of the samples. Resistance to the 4 antifungal agents ("antifungaltype" 2222) was detected in 1.0% of the isolates (Table 2).

The same "antifungaltype" was found in different biological materials of a single patient and in a given type of clinical specimen recovered from the same patient during different episodes (Table 3).

DISCUSSION

The *in vitro* susceptibility of microorganisms to chemotherapeutic agents is used to guide treatments (20). When the responses of at least 3 drugs are grouped, "models", "types" or "patterns" can be

Table 1. *In vitro* inhibitory activities (μg/mL) of four antifungal agents against 100 *C.albicans* isolates determined by the agar dilution method.

DRUG	RANGE	MIC[a]	MIC_{50}[b]	MIC_{90}[c]
Amphotericin B	0.25 - 2.0	1.17	1.0	1.0
Ketoconazole	0.12 - 32.0	9.8	16.0	32.0
Miconazole	0.12 - 32.0	6.8	8.0	32.0
Flucytosine	0.12 - > 128.0	1.4	0.5	> 128.0

a Geometric mean of minimum inhibitory concentration.
b Concentration at wich 50% of the isolates are inhibited.
c Concentration at wich 90% of the isolates are inhibited.

Table 2. Distribution of 12 "antifungaltypes" identified among 100 *C.albicans* strains.

Number of strains	"antifungaltypes"
1	2212 / 2222
3	1122 / 2111
5	1212 / 2221
6	1112 / 1222
10	1111
15	1121
20	1221
25	1211

Discriminatory index = 0.8

Table 3. Distribution of the "antifungaltypes" of 12 *C.albicans* strains from 5 patients, according to the origin of the clinical specimen.

Patients	*C.albicans*	Clinical specimen	"Antifungaltype"
1	1a	sputum	1211
	1b	oropharingeal swab	1211
2	2a	sputum	1121
	2b	bronchial washing	1121
3	3a	bronchial washing	2221
	3b	bronchial washing	2221
4	4a	skin lesions	1211
	4b	skin lesions	1211
	4c	skin lesions	1211
	4d	skin lesions	1211
5	5a	blood culture	1211
	5b	blood culture	1211

established which play an important role in epidemiological surveys or in therapeutic monitoring.

In order to determine the levels of resistance of *C.albicans* strains to the antifungal agents, serum drug levels were taken into consideration before setting up test concentration ranges (4, 16).

Ten percent (10.0%) of the *C.albicans* strains were resistant to amphotericin B. Rates of 0.8 to 83.3% have been reported by different investigators (2, 4, 5).

The results for ketoconazole and miconazole were similar, with 63.0 and 50.0% resistant cultures, respectively. The values reported in the literature for these drugs range from 0.0 to 80.0% (1,2).

Flucytosine at the concentration of 8.0 μg/mL inhibited 78.0% of the strains, which agrees with data reported by other authors (18).

The use of a digital system for MIC values in the sequence amphotericin B> ketoconazole> miconazole> flucytosine revealed 12 "antifungaltypes" or models (Table 2), with prevalence of types 1211 (25.0%) and 1221 (20.0%).

The "antifungaltype" coded by 1111 was detected in 10.0% of the strains (Table 2). This type, which denotes susceptibility to the four antifungal agents, was observed by Bergan and Vangdal (2) in 100.0% of their strains. The difference in frequency may perhaps be attributed to the concentrations of inocula, since the inoculation system used in both studies was similar. Suspensions containing approximately 1-2 x 10^6 cells/mL were used in our investigation, as recommended by Shadomy *et al.* (19), whereas suspensions of 1 x 10^5cells/mL were used by Bergan and Vangdal (2). In our study the standard strains were sensitive to the test concentrations.

Differences between the population groups, regional characteristics, acquired resistance and different methodologies are other factors that should be taken into consideration.

"Antifungaltyping" revealed a good discriminatory index, similar to those observed by some investigators for other typing techniques (8).

The present results demonstrate that "antifungaltyping" is an important tool as epidemiological marker and should be applied to routine laboratory procedures to establish chemotherapeutic protocols. Coding of the results as a digital system to form "antifungaltypes" facilitates the interpretation of data, treatment monitoring and the establishment of epidemiological correlations. Further studies with a larger number of antifungal agents, including new drugs, should be performed to improve the discriminatory power of the technique.

RESUMO

Candida albicans: Biotipagem pela susceptibilidade aos antimicóticos

Foi avaliada a atividade *in vitro* de anfotericina B, cetoconazol, miconazol e fluorocitosina, frente a 100 amostras de *C.albicans* isoladas de diferentes espécimes clínicos. Na concentração de 1,0 µg/mL a anfotericina B foi o agente mais efetivo, inibindo 90,0% de todas as amostras, o cetoconazol na concentração de 8,0 µg/mL foi o menos efetivo, inibindo somente 37,0% dos isolamentos de *C.albicans*. Susceptibilidade ou resistência aos 4 agentes antifúngicos foram detectadas em 10,0% e 1,0% das amostras, respectivamente. Baseado na susceptibilidade a anfotericina B, cetoconazol, miconazol e fluorocitosina, foi desenvolvido um sistema digital denominado de "antifungotipagem", o qual permitiu identificar 12 diferentes tipos, modelos ou padrões. O biotipo 1211 foi o mais freqüentemente isolado, seguido do tipo 1221 (20%). As amostras isoladas do mesmo paciente em diferentes ocasiões sempre apresentaram o mesmo tipo, como 1211 de lesão de pele e 2211 de lavado brônquico, ou do mesmo paciente e da mesma ocasião mas de diferentes espécimes clínicos, como os tipos 1211 (escarro e orofaringe) e 1121 (lavado brônquico e escarro).

Palavras-chave: *Candida albicans*, biotipagem, anfotericina B, cetoconazol, miconazol, fluorocitosina.

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YEASTS VECTORED BY *DROSOPHILA QUADRUM* (*CALLOPTERA* GROUP) IN TROPICAL RAIN FORESTS

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ABSTRACT

Yeast-*Drosophila* interactions in Tropical Rain Forests of Brazil are specialized when compared to temperate ecosystems, and tropical species of *Drosophila* have preferences that vary in the degree of choice of yeasts. Yeasts associated with *Drosophila* of the forest-inhabiting *tripunctata, willistoni* and *guarani* groups are probably of fruit origin. They differ from yeasts isolated from the *fasciola* subgroup flies of the *repleta* group, which seem to colonize epiphytic cacti in the forest canopy. The yeasts vectored by *Drosophila quadrum* (*calloptera* group) were surveyed and compared with the communities associated with the flies of *tripunctata, willistoni, guarani* and the cosmopolitan *melanogaster* group in forest sites of Rio de Janeiro. The yeasts vectored by *D. quadrum* included *Candida guilliermondii, Debaryomyces melissophilus, Debaryomyces vanriji, Kloeckera apis, Pichia membranaefaciens* and *Rhodotorula rubra* as most frequent species. These yeasts are usually associated with flowers and deteriorating fruits in the forest, indicating that *D. quadrum* feeds preferably on flowers and fruits in advanced states of decomposition. The yeasts associated with the *calloptera* group were similar to yeasts isolated from the *tripunctata* and *guarani* flies that probably occupy similar niches on the forest floor and vicinities. The *calloptera* flies had a lower niche overlap with flies from the *fasciola* subgroup, and from the *willistoni* and *melanogaster* groups in the same forest.

Key words: yeasts, *Drosophila*, habitat choice, diversity.

INTRODUCTION

The mutual interactions of yeasts and *Drosophila* provide a model system where the yeasts are dependent on the flies for dispersion to new environments and provide nutrients and breeding stimuli for the drosophilids (22). Natural environments are spatially heterogeneous, and *Drosophila* species respond to this microgeographic variation, both genetically and behaviorally (20, 21, 23, 24). Drosophilids feed and breed in sites which are discrete and ephemeral, supporting a large number of species even though there is little evidence of resource partitioning (17). The yeast micota used as food by the flies seems to be involved in the structuring of *Drosophila* communities in savannas and adjoining gallery forests, in cactus substrates, and also in Atlantic Rain Forests (3, 9, 11, 12). The yeasts

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vectored by *Drosophila* in the Tropical Rain Forests of Southeastern Brazil showed high species diversity, and specificity of association with the flies (11). Yeasts associated with typical forest-inhabiting *Drosophila* of the groups *tripunctata, willistoni* and *guarani* and also the yeasts vectored by invader *melanogaster* flies were probably of fruit origin (11). Differences in the yeast communities among the fly groups suggested a differentiation of diets and specialization of the yeast-*Drosophila* associations in the tropical forests (11). Da Cunha *et al.* (2) have shown that each *Drosophila* species has varying degrees of preference in choice of yeasts. The forest-dwelling (ground feeders) *Drosophila calloptera* group breeds and feeds on small dried fruits and blossoms on the forest floor, and responds to seasonal fluctuations of the resources. This behavior is similar to that of the *tripunctata* and *cannalinea* groups and to that of the drosophilid genus *Clastopteromyia*. The *calloptera* group differed from the *willistoni* group that uses dry and fleshy fruits and does not show seasonal population fluctuations. It also differed from the cosmopolitan *melanogaster* group, which chiefly uses fleshy fruits and has population expansions during the dry spring fruiting months (17). We report the vectoring of yeasts by *Drosophila quadrum* (*calloptera* group) in the Atlantic Rain Forests of Rio de Janeiro, and compare the yeast species composition and diversity with those of the *tripunctata, guarani, willistoni,* and *melanogaster* groups surveyed in the same region (11) and also with the yeast community associated with the *fasciola* subgroup of the cactiphilic *Drosophila repleta* group in these forests (13).

MATERIALS AND METHODS

The collections were done at the following sites of the State of Rio de Janeiro: Tijuca National Park forest, in December of 1989, January and August of 1990, June and August of 1991; Itacoatiara beach forest, in June and July, 1991; and the Rio Bonito (Nova Friburgo) forest, in March, 1992. The procedures for collection and isolation of yeasts vectored by the *Drosophila calloptera* followed Morais *et al.* (11). The flies were attracted to banana baits fermented overnight with a mutant strain OLI R56 Ad$^-$ of *Saccharomyces cerevisiae* (obtained from Dr. A. Panek, Instituto de Química of the Universidade Federal do Rio de Janeiro) that forms easily identifiable red pigmented colonies on isolation medium (YM agar: yeast extract 0.3%; malt extract 0.3%; peptone 0.5%; glucose 1%; agar 2%; adjusted to pH 3.7-4.0 with 0.7% of 1N HCl and supplemented with 100 mg/L of chloramphenicol). The bait was covered with sterile gauze layers to avoid contamination of flies with the yeasts from the bait. The flies of the *calloptera* group were usually captured on plant surfaces and forest floor near the bait. In the Rio Bonito collection, the flies were captured with a compartmentalized trap that was hung on tree branches three to seven meters high in the forest canopy. The trap used mashed banana fermented overnight with the mutant strain OLI 1 R56 of *S. cerevisiae* for attraction of flies (13). The flies were allowed to walk for eight to 12 hr on plates of isolation medium in order to recover yeasts vectored on the surface of the insects. The yeasts isolated by this procedure could include regurgitated cells, from fecal pellets, or contaminants from the ovipositor of female flies. These flies were then recovered for identification. Another set of flies was transferred to alcohol 70% for one minute for surface disinfection and transported to the laboratory, within two hours, in tubes kept on ice and containing sterile distilled water. These were identified and the crops aseptically dissected from them were streaked on YM agar for isolation of yeasts used as food by the flies. Plates were incubated at 25 ± 3°C and the yeasts isolated. Yeast isolates were characterized and identified according to standard methods (1, 7, 25). The flies were identified according to Vilela and Bächlii (26). The diversity index was calculated as the odds measure of diversity (8). The measure of niche breadth of Hulbert (5) was used to obtain the feeding niche of *Drosophila quadrum* and the other *Drosophila* groups. The similarity among the *Drosophila* groups determined by the Morisita's similarity index (15) was considered a measure of niche overlap (6).

RESULTS AND DISCUSSION

The prevalent yeasts associated with *Drosophila quadrum* belonged to *Debaryomces melissophilus* and *Debaryomyces vanrijae* and summed about 20 % of the isolates (Table 1). Other yeasts associated with *D. quadrum* are usually isolated from flowers, like *Candida guilliermondii* and *Kloeckera apis*, and still others like the black yeasts, *Candida antarctica,* and *Rhodotorula rubra* are typical of plant surfaces (18, 19). *Candida diversa, Candida insectamans, Candida quercitrusa, Debaryomyces*

Table 1. Yeasts isolated from *Drosophila quadrum* in three forests of Rio de Janeiro.

Yeasts	Tijuca Forest		Itacoatiara Forest		Rio Bonito Forest
	WP[a] (n=27)[c]	CROP[b] (n= 2)	WP (n=44)	CROP (n=19)	WP (n=11)
Black yeasts	5		3		2
Candida antartica			1		
Candida apicola	1		5	1	
Candida apis var. *galacta*	3				
Candida bimundalis	1	1	1		
Candida blankii					1
Candida colliculosa	1				
Candida diversa	1		4		
Candida fructus		1	6		
Candida guilliermondii	2		10	3	1
Candida insectamans			1		
Candida intermedia					1
Candida lambica	1				
Candida melinii		1			1
Candida pelliculosa	3				
Candida quercitrusa	2	1	7	1	
Candida sorboxylosa			1		
Cryptococcus albidus					1
Cryptococcus humicolus			1		
Cryptococcus hungaricus	1		3		
Cryptococcus luteolus	1		4	2	1
Debaryomyces melissophilus	1		9	3	4
Debaryomyces vanriji	12	2	12	2	
Geotrichum sp.	1	1	6	2	1
Hanseniaspora uvarum			1	1	
Issatchenkia occidentalis			1	1	
Kloeckera africana			1		
Kloeckera apis	3		10	2	3
Kloeckera japonica	1				
Kloeckera javanica	1				
Kluyveromyces marxianus var. drosophilarum			1		
Pichia acaciae	3		2	4	
Pichia anomala	1				
Pichia beckii	1	1			
Pichia kluyveri		1			
Pichia lindneri	1		3		
Pichia membranaefaciens	2		8		
Rhodotorula glutinis	1		3		
Rhodotorula rubra	3		9	1	4
Torulaspora delbrueckii			1	4	
TOTAL	53	9	114	27	20

[a] WP: walking procedure which represents yeasts carried on the external surfaces of the flies.
[b] CROP: yeasts isolated from dissected crops of the flies.
[c] n: number of flies.

vanrijae, *Pichia membranaefaciens* and its anamorph *Candida valida*, also isolated from *D. quadrum*, are similar to those associated with the late decomposing stage of amapa fruits in the Amazon forest (14). The presence of these yeasts indicates that flowers and deteriorated fruits are the resources utilized by flies of the *calloptera* group in these forests. The yeasts *Candida intermedia*, *Candida krusei*, *Hanseniaspora uvarum* and *Pichia membranaefaciens* were isolated from *Drosophila atrata* and *Drosophila quadrum* in the Atlantic Rain Forests of São Paulo, Brazil (2). Similarly, we have isolated *C. intermedia*, *C. sorboxylosa* (which presents a similar physiological profile to *C. krusei*), *Hanseniaspora uvarum*, and *P. membranaefaciens* from *D. quadrum*, evidencing the fidelity of the *calloptera* flies to their microhabitat in the Tropical Rain forests.

The differences in microbiota used as food by Neotropical *Drosophila* species would explain the successful coexistence of different species. The Neotropical species of *Drosophila* regularly share the same adult feeding and breeding sources. They are usually found as aggregations of many species belonging to the same species group and even to the same sibling sets on a variety of fallen fruits and blossoms (4, 17). Mechanisms such as diet preferences and seasonal fluctuations noted among forest groups of *Drosophila* have been linked to choice of different yeasts and may serve to avoid competition among the flies (10, 14). Three overlapping classes of Neotropical forest-dwelling Drosophilidae can be defined according to fruit preferences and attractability to traps baited with cultivated fruits (17). The class A flies include species with wet expansions that survive the dry season by breeding in living fruits. The B and C classes show both dry and wet expansions or absence of seasonal fluctuations, the cosmopolitan class C expanding significantly during fruiting months. The *calloptera* and *tripunctata* groups belong to class A, and show a large niche overlap (Table 2) and preference for *D. vanrijae* as the most frequently associated yeast (Table 1 and ref.11). The feeding niche of *D. quadrum* was much narrower than the niche of the other forest-dwelling *Drosophila*, including the *tripunctata* flies (Table 2). Probably, the *tripunctata* group is more of a generalist than the *calloptera* group. It seems to share microhabitats with the *guarani, willistoni* and *melanogaster* group to some extent, as indicated by the high measure of niche overlap among them (Table 2 and ref.11). The niche overlap between *D. quadrum* and the *guarani* group was also significantly higher than the overlap with *willistoni* and *melanogaster* flies. These last flies are associated with fermentative apiculate yeasts known to be responsible for the initial souring of sugary fruits, and rarely isolated from *D. quadrum* (14, 16). The yeasts associated with the flies of the *guarani* group include the black yeasts, *Candida apicola, Geotrichum* sp., and *Kloeckera apis*, which were also isolated from *D. quadrum* and would probably account for the degree of overlap between them (Table 1, ref. 11). The yeast community of *calloptera* flies showed a low degree of overlap to the yeast community associated with the *fasciola* subgroup, which prefers cactus substrates in the forest canopy and has a narrow feeding niche, indicating a high degree of habitat specialization (13). The feeding niche of *D. quadrum* was probably limited to the forest floor and vicinities, where this fly species visited and fed on yeasts colonizing fruits in late states of decomposition and flowers. The *tripunctata* group may include various species that overlapped in niche with the *calloptera* group, and also visited other substrates utilized mostly by the *willistoni* and *guarani* groups. The recently fallen fruit microhabitat of the yeasts associated with the *willistoni* and *melanogaster* flies was apparently not used by *D. quadrum*, which also seems to avoid the forest canopy habitat typical of the *fasciola* subgroup. The habitat heterogeneity provided by the variety of yeast-colonized substrates seems to provide different habitats for the drosophilids. This should lower the effects of interspecific competition where substrates and fly species are abundant and diverse.

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Table 2. Yeast diversity, feeding niche and diet overlap of *Drosophila quadrum* and other forest *Drosophila* groups

Host	Yeast diversity	Feeding Niche	Diet Overlap				
			D. quadrum	tripunctata	guarani	willistoni	melanogaster
Drosophila quadrum	15,34	0,24					
tripunctata GROUP	12,30	0,77*	0,588				
guarani GROUP	9,40	0,60*	0,319	0,229*			
willistoni GROUP	19,40	0,55*	0,251	0,309*	0,229*		
melanogaster GROUP	3,70	0,56*	0,058	0,148*	0,000*	0,274*	
fasciola SUBGROUP	8,70	0,13*	0,125	0,002*	0,003*	0,002*	0,005*

RESUMO

Leveduras dispersadas por *Drosophila quadrum* (grupo *calloptera)* em florestas tropicais

As interações levedura-*Drosophila* em florestas tropicais no Brasil são especializadas quando comparadas com ecossistemas temperados e espécies tropicais de *Drosophila* possuem graus diferentes de preferência por leveduras. Leveduras associadas a *Drosophila* dos grupos florestais *tripunctata, willistoni* e *guarani* são provavelmente originárias de frutos, e diferem de leveduras isoladas de moscas do subgrupo *fasciola* do grupo *repleta* que colonizam cactos epifíticos no extrato arbóreo da floresta. As leveduras dispersadas por *Drosophila quadrum* (grupo *calloptera*) foram estudadas e comparadas com as comunidades associadas a moscas dos grupos florestais *tripunctata, willistoni, guarani* e o cosmopolita *melanogaster* em sítios florestais do Rio de Janeiro. As leveduras vetorizadas por *D. quadrum* incluíram *Candida guilliermondii, Debaryomyces melissophilus, Debaryomyces vanriji, Kloeckera apis, Pichia membranaefaciens* e *Rhodotorula rubra* como espécies mais freqüentes. Estas leveduras são geralmente associadas com flores e frutos apodrecidos na floresta, indicando que *D. quadrum* alimenta-se preferencialmente em flores e em frutos em avançado estado de decomposição. As leveduras associadas com o grupo *calloptera* mostraram similaridade com leveduras isoladas de moscas dos grupos *tripunctata* e *guarani* que provavelmente ocupam nichos similares no solo da floresta e arredores. As moscas *calloptera* mostraram baixa sobreposição de nicho alimentar com as moscas do subgrupo *fasciola* e grupos *willistoni* e *melanogaster* nas mesmas áreas florestais.

Palavras-chave: levedura, *Drosophila*, escolha de habitat, diversidade.

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EFFECT OF NITRATE ON NODULATION AND N_2-FIXATION OF *LUPINUS MUTABILIS* MUTANTS AT DIFFERENT GROWTH STAGES [1]

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ABSTRACT

The inhibitory effect of nitrate on biological dinitrogen fixation by *Rhizobium*-legume symbiosis is well known, but the mechanisms involved are still not clear. One of the main techniques used to study this effect has been the use of mutants of *Rhizobium* strains or host plants which do not express the usual pattern of nodulation. The present work was conducted using parental genotypes and mutants of *Lupinus mutabilis* with hypernodulation or low nodulation phenotypes that were submitted to two levels of nitrate (0 and 5 mM) and harvested at two different times (7 and 10 weeks after emergence). The five millimolar concentration appeared to be very important for inhibition of the initial nodulation of *Lupinus*, although mutants L-114 and L-105 were more efficient in nodulation, N-yield and nitrogenase activity than the cv. 'SCG-25' and may allow increased nitrogen fixation in the presence of nitrate. There were no differences in the GS activity of nodules among L-114, L-105 and cv. 'SCG-25' when the plants were grown without nitrate, yet there was a tendency for mutant L-114 to show higher GS activity in the presence of nitrate. The results indicate that mutant L-114 is more capable of assimilating fixed nitrogen, as also suggested by its PEP-carboxilase activity. Thus, the partial tolerance to nitrate presented by L-114 and L-105 is apparently associated with the hypernodulated phenotype. This is in fact illustrated in the results of total and specific acetylene reduction activity, which were higher for mutant L-114 than for cv. 'SCG-25' both in the absence and presence of nitrate.

Key words: *Bradyrhizobium*, glutamine synthetase, phosphoenolpyruvate carboxilase, nodule cytosol, N-metabolism, acetylene reduction.

INTRODUCTION

The general inhibitory effect of nitrate (NO_3^-) on nodulation and N_2 fixation in legume-rhizobia symbiosis has been the subject of intensive research for many years. However, the mechanisms of biochemical-physiological NO_3^- inhibition on legume nodulation and N_2 fixation are still unknown (24). Other results have shown that NO_3^- limits the infection process, the development of nodules and the

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subsequent expression of nitrogenase activity in the bacteroids and also hastens the breakdown of nodule tissues (9, 13).

The influence of nitrate remains unclear, especially during the nodulation stages, where it is not known whether the effects are due to the external presence of NO_3^- or to the events that follow uptake and/or metabolism. Recently, Cho and Harper (6) showed that the site of N-application primarily controls inhibition of nodulation, possibly by a decrease in the internal levels of isoflavonoids in the root. The authors also presented evidence that NO_3^- has a systemic effect on root isoflavonoid compounds. A direct effect of NO_3^- on nodulation has been indicated by split-root studies with *Glycine max* (L.) Merr. and by excised root experiments with *Phaseolus vulgaris* (L.). In both cases, Raggio *et al.* (21) reported a positive interaction of NO_3^- and carbohydrates with rhizobial root nodules formation. In another work, the incorporation of NH_4NO_3 (crown region) was more inhibitory to nodulation than was its placement below this zone (15).

Fenstra *et al.* (10) described a partially nitrate-reductase deficient mutant of *Pisum sativum* (L.) less susceptible to the effects of NO_3^- on symbiotic N_2 fixation than the wild type. In contrast, Ryan *et al.* (20) reported that a nitrate-reductase deficient soybean mutant did not show improved nodulation compared with the wild type, supporting the suggestions that NO_3^- metabolism was involved. E. Jacobsen personal communication to Gibson and Harper (11) showed a second type of pea mutant with a greater tolerance to NO_3^- during nodulation, despite having normal nitrate-reductase activity. These observations indicate that the adverse effects of NO_3^- on nodulation can be overcome by other mechanisms, such as limited carbon supply to the nodule rather than by the altered NO_3^- metabolism of cultivars.

On the other hand, work done by Gibson and Harper (11) with two soybean genotypes and one *Bradyrizobium japonicum* strain led to the conclusion that manipulation of the host plant rather than the bacterial strain was the primary control in alleviating the negative impact of NO_3^- on the level of N_2 fixation.

Several laboratory groups have attempted to induce variability in the host nodulation response, and supernodulating mutants have been generated in soybean (3, 14), white bean (19) and pea (17). These mutants are useful for determining which plant factors are associated with the regulation of nodule numbers, nitrate effects on infection and N_2 fixation. While the supernodulating genotypes of other legumes have been well characterized (2, 8, 14), similar information is not available for *Lupinus sp.* Thus, the aim of this study was to compare selected physiological parameters of *Lupinus* mutants lines with their wild type parents under different levels of nitrate and growth stages.

MATERIALS AND METHODS

The experiment was conducted on a completely randomized block design, with four replications. Treatment combinations were formed by a factorial with six genotypes (cultivars '176', 'SCG-25' and mutants L-114, L-109, L-123, and L-57), two growth stages (7 and 10 weeks), and two nitrate levels (0.0 and 5.0 mM). F-tests (0.05 level of significance) were based on ANOVA. For the significant F-tests, LSD values were calculated for multiple comparisons between two treatment means.

Seeds of *Lupinus mutabilis* cultivars and selected nodulation mutants (Table 1) were disinfected by immersion in ethanol 70% for 20 min. and then planted in 2.5 L Leonard jars using perlite as growth medium. After uniform germination of seedlings, three plants per jar were left as the experimental material. The plants were inoculated twice at planting and one week later with a mixture of strains L-182 and L-750 of *Bradyrhizobium* sp. (*Lupinus*). The experiment was conducted during the 1992 Spring in a greenhouse under solar photoperiod, at 30°C during daytime and 15°C at night. A N-free solution (4) was periodically supplied to the jars, and KNO_3 at 5mM N was added to the solution for nitrate treatment. Readditions of nutrient solution and KNO_3 were necessary for maintenance of the rooting medium as well as the combined N at the desired level.

The plants were harvested at two growth stages, namely: 7 and 10 weeks after emergence. At each

Table 1. Characteristics of the E.M.S. (Ethyl-methane sulfonate at 0.04 M) *Lupinus mutabilis* mutants used in the experiment.

Cultivar[a]	Mutant Lines	Selection Year	Initial Symbiotic Characteristics
176	123	1988	Low nodulation
SCG-25	57	1988	Low nodulation
	105	1987	Hyper nodulation
	114	1988	Hyper nodulation

a Cultivars '176' and 'SCG-25', originated from the Cuzco Collection Cultivars, Peru, were also used as control

harvest, nitrogen fixation for each treatment was determined by standard acetylene (C_2H_2) reduction assays of the whole root system using a FI detector and a Poropak-R column in Schimadzu GC, as described by Chamber-Perez and Iruthaythas (5). Shoots were dried in an oven at 70°C for 48 h and their total nitrogen determined with an Infralyzer Technicon 300B.

Number and fresh weight of nodules were determined and 1.5g of the nodules from each treatment were homogenized under N_2 stream at 4°C in phosphate buffer (23). The homogenate solution was passed through a cheese-cloth layer and the suspension was collected in tubes centrifuged next at 200g for 5 min. The supernatants obtained were recentrifuged at 8,000 g. for 20 min at 4°C, to separate bacteroids from cytosol. Soluble protein was measured according to Goa (12) and samples of cytosol suspension were analyzed for nodule glutamine synthetase (GS) (EC 6.3.1.2.) and phosphoenolpyruvate carboxilase (PEPC) (EC 4.1.1.31) activities. Nodule GS activity was determined using the ADT-transferase reaction that measures the formation of *y*-glutamylhidroxamate (22), while nodule PEPC activity was determined according to the method described by Briand *et al.* (1).

RESULTS AND DISCUSSION

The application of five millimolar nitrate resulted in inhibition of nodulation in both *Lupinus* wild types and their mutants when compared with plants which did not receive nitrate (Tables 2 and 3). The inhibitory effect of nitrate on nodule number and nodule fresh weight was more evident in the cvs. 'SCG 25' and '176' than in mutants L-114, L-105 and L-57. Nodule mass increased especially in the later stage of growth, but this trend was less evident for nodule numbers when comparing wild type parent cv. 'SCG-25' with their mutants L-57, L-105 and L-114. The combined analyses of variance between N-levels and growth stages for both parameters of nodulation showed that the performance of mutant L-123 was inferior to that of the parental cv. '176'.

Concerning nodulation inhibition and subsequent nitrogenase activity, the results generally agree with previous data on these parameters associated with nitrate addition. However, it is necessary to emphasize the work of Wu and Harper (25), which demonstrated that nodulation of soybean mutants was less sensitive to nitrate application than nodulation of the original genotype; their data confirmed that nodulation and nitrogen fixation were only partially affected, since the ureide abundance in shoots was different when comparing mutants with their parents. Some mutants showed a fresh nodule weight gain, suggesting a certain relationship with the previously noted hypernodulant character. Other reports indicated that *Pisum sativum* mutants with deficient nitrate reductase were less sensitive to nitrate application than their parents (10). Nevertheless, soybean nitrate reductase deficient mutants did not show improved nodulation under nitrate application when compared to the wild type (11) suggesting that the adverse effects of nitrate application on nodulation have various causal factors,

Table 2. Effects of nitrate levels and growth stages on nodule number of six *Lupinus mutabilis*: two cultivars as control (cvs.'176', 'SCG-25'), two hypernodulating mutants (L-114, L-105) and two low-nodulating mutants (L-123, L-57)[1]

		Nodule number (plant^{-1})[2]							
		cv.'176'	cv.' SCG-25'	L-114	L-105	L-123	L-57	Mean	LSD[3]
N	0mM	82.9	78.8	42.0	83.1	38.7	59.5	64.2	7.7
level	5mM	45.0	35.5	30.9	76.8	27.1	35.3	41.8	
	LSD[4]	18.85							
Growth	7 weeks	47.3	52.2	31.0	73.6	27.7	40.9	45.4	NS
Stage	10 weeks	80.6	62.1	42.0	86.5	38.2	53.9	60.5	
	LSD[5]	NS							
	Overall Mean	63.9	57.1	36.5	79.9	32.9	47.4		
	LSD[6]	NS							

[1] - Hypernodulating and low nodulating mutants are designated according to a previous selection work done in 1987 and 1988.
[2] - Each value is an average of 12 plants contained in four Leonard jars with 3 plants. jar^{-1}.
[3] - LSD (0.05), between N levels and growth stages, averaged across *Lupinus* genotypes.
[4] - LSD (0.05), between two N levels, within a *Lupinus* genotype.
[5] - LSD (0.05), between two growth stages within a *Lupinus* genotype.
[6] - LSD (0.05), between *Lupinus* genotypes, averaged across N levels and growth stages.

which limited the carbon supply to nodules and altered nitrate metabolism.

In the first growth stage (7 weeks), only slight differences in dry weight were apparent between the mutant lines (Table 4). However, the low-nodulating mutant L-123 without nitrogen showed small evidence of nitrogen deficiency and the dry matter yield from this treatment was very low. In the presence of combined nitrogen, substantial differences between mutant L-105 and the other three nodulating genotypes became apparent at both stages of growth (7 and 10 weeks), with the hypernodulating mutant producing approximately the same amount of dry matter as the parental cultivar cv. "SCG-25" and of as the other wild type, cv. '176'.

Similar results were observed at the second harvest for mutant L-105, although the plant dry weights of mutants L-114 and L-123 were similar. Shoot dry weight of mutant L-114, initially considered hypernodulating, was similar to the low nodulating mutant L-57 at the same N level and also at different growth stages. The five millimolar nitrate treatments increased the growth of all genotypes. For mutant L-105, however, the increase in biomass production was significantly higher than that of the other mutants and wild types. Combined analyses of variance for shoot dry weight across N levels and growth stages showed that mutant L-105 accumulated dry weight in the same amount as did wild type and more than the other mutant lines.

Table 3. Effects of nitrate levels and growth stages on nodule fresh weight of six *Lupinus mutabilis*: two cultivars as control (cvs.'176', 'SCG-25'), two hypernodulating (L-114, L-105) and two low-nodulating mutants (L-123, L-57)[1]

		Nodule fresh weight (g.plant^{-1})[2]							
		cv.'176'	cv.' SCG-25'	L-114	L-105	L-123	L-57	Mean	LSD[3]
N	0mM	0.98	0.80	0.82	0.61	0.30	0.98	0.75	0.08
level	5mM	0.39	0.34	0.40	0.48	0.31	0.30	0.37	
	LSD[4]	0.19							
Growth	7 weeks	0.34	0.27	0.30	0.30	0.21	0.35	0.30	0.09
Stage	10 weeks	1.03	0.87	0.93	0.79	0.41	0.93	0.82	
	LSD[5]	0.19							
	Overall Mean	0.68	0.57	0.61	0.55	0.31	0.64		
	LSD[6]	0.28							

1 - Hypernodulating and low nodulating mutants are designated according to a previous selection work done in 1987 and 1988.
2 - Each value is the average of 12 plants contained in four Leonard jars with 3 plants. jar^{-1}.
3 - LSD (0.05), between N levels and growth stages, averaged across *Lupinus* genotypes.
4 - LSD (0.05), between two N levels, within a *Lupinus* genotype.
5 - LSD (0.05), between two growth stages within a *Lupinus* genotype.
6 - LSD (0.05), between *Lupinus* genotypes, averaged across N levels and growth stages.

Table 4. Effects of nitrate levels and growth stages on shoot dry weight of six *Lupinus mutabilis*: two cultivars as control (cvs.'176','SCG-25'), two hypernodulating (L-114, L-105) and two low-nodulating mutants (L-123, L-57)[1]

		Shoot dry weight (g.plant^{-1})[2]							
		cv.'176'	cv.' SCG-25'	L-114	L-105	L-123	L-57	Mean	LSD[3]
N	0mM	1.37	1.30	0.91	0.98	0.49	1.02	1.01	0.16
level	5mM	2.48	2.52	1.66	2.70	1.55	2.06	2.16	
	LSD[4]	0.40							
Growth	7 weeks	1.09	1.20	0.74	0.98	0.72	0.94	0.95	0.22
Stage	10 weeks	2.76	2.62	1.83	2.69	1.32	2.14	2.23	
	LSD[5]	0.16							
	Overall Mean	1.92	1.91	1.29	1.84	1.02	1.54		
	LSD[6]	0.32							

1 - Hypernodulating and low nodulating mutants are designated according to a previous selection work done in 1987 and 1988.
2 - Each value is an average of 12 plants contained in four Leonard jars with 3 plants. jar^{-1}.
3 - LSD (0.05), between N levels and growth stages, averaged across *Lupinus* genotypes.
4 - LSD (0.05), between two N levels, within a *Lupinus* genotype.
5 - LSD (0.05), between two growth stages within a *Lupinus* genotype.
6 - LSD (0.05), between *Lupinus* genotypes, averaged across N levels and growth stages.

The total N yields of the different genotypes are shown in Table 5. There was a marked increase in total shoot nitrogen when nodulated plants were supplied with 5.0mM of NO_3^-. A great change occurred in mutants L-105 and L-123, where their respective N yields were approximately 3.8 and 4.4 times higher than those for the fully symbiotic plants. When plants grew up to 10 weeks, the N yield of mutant L-105 was slightly greater compared with the wild type genotype, although the difference was significant with respect to the other mutants L-114, L-123, and L-57. Similar results were observed in the combined analyses across N levels and growth stages.

The increase in accumulation of total N with the application of nitrate was an expected result. Herridge and Brockwell (16), however, observed that nitrate applications of 100 and 200 kg N/ha substantially repressed N_2 fixation by wild type soybean cv. Bragg in the field, but were insufficient at normal rates of inoculation. They found an N yield reduction of approximately 35% in comparison to the 0 N level. This does not agree with the observation presently reported that mutant L-105 maintained a high N yield in the presence of 5mM of NO_3^-. Carrol *et al.* (2) reported that the nitrate reductase results for leaves of the nitrate tolerant symbiosis-mutant 382 of soybean were similar to those for the cv. Bragg, yet there are no indications of this activity in the roots of mutant 382. Thus, the nitrate tolerance of this mutant is due to a lack of nitrate reduction in the roots. The results in Table 5 suggest that the *Lupinus* mutants tested, L-114 and L-105, were partially tolerant to NO_3^-. This result might be confirmed by other tests like the nitrate reductase activity in the roots, as speculated by Crafts-Brandner and Harper (7) who reported that the greater tolerance of soybean mutants to nitrate is related to a higher nitrate reductase activity found in the roots. The evidence from our data is insufficient to support this cause and effect relationship.

Addition of NO_3^- to nodulated *Lupinus* plants resulted in decreased nitrogenase activity (C_2H_2 reduction) in all four mutants and in the wild type parents (Tables 6 and 7). The nitrogenase activity of the mutants was similarly affected by NO_3^- and the cv.'176' and cv.'SCG-25' were strongly inhibited by nitrate. In the absence of NO_3^-, the hypernodulating mutant L-114 had greater nitrogenase activity than its parental cv. 'SCG-25'. Otherwise, mutants L-105, L-123, and L-57 showed lower nitrogenase activity in comparison with the wild type plants. The C_2H_2 reduction assays clearly showed that all genotypes were sensitive to the added NO_3^-, but the hypernodulating mutant L-105 exhibited partial tolerance to nitrate, mainly in the earlier stages of N_2 fixation, compared with cv. 'SCG-25'. With respect to the hypernodulating mutant L-114, the nitrogenase activity showed that this genotype is more sensitive to NO_3^-, with a delayed initiation of N_2 fixation when compared to the wild type parent.

Acetylene reduction per plant on medium without nitrate was higher in the mutant L-114 than in cvs. 'SCG-25' and '176', although the increase was less than that for the fresh nodule weight per plant, or nodule number. Otherwise, the specific C_2H_2 reduction activity in mutant L-114 was about 100% more than that of cvs. 'SCG-25'and '176'. These results suggest that the insufficient supply of carbohydrates by the leaves associated with an

Table 5. Effects of nitrate levels and growth stages on N-yield of six *Lupinus mutabilis*: two cultivars as control (cvs.'176', 'SCG-25'), two hypernodulating (L-114, L-105) and two low-nodulating mutants (L-123, L-57)[1]

		N yield (g.plant^{-1})[2]							
		cv.'176'	cv.' SCG-25'	L-114	L-105	L-123	L-57	Mean	LSD[3]
N	0mM	38.45	36.35	24.47	24.98	11.51	25.88	26.94	7.88
level	5mM	84.38	88.13	61.13	95.30	51.35	72.52	75.47	
	LSD[4]	14.45							
Growth	7 weeks	36.58	39.62	25.28	32.13	24.11	29.67	31.23	5.90
Stage	10 weeks	86.25	84.87	60.32	88.15	38.75	68.73	71.18	
	LSD[5]	19.30							
	Overall Mean	61.42	62.24	42.80	60.14	31.43	49.20	49.20	
	LSD[6]	NS							

1 - Hypernodulating and low nodulating mutants are designated according to a previous selection work done in 1987 and 1988.
2 - Each value is an average of 12 plants contained in four Leonard jars with 3 plants. jar^{-1}.
3 - LSD (0.05), between N levels and growth stages, averaged across *Lupinus* genotypes.
4 - LSD (0.05), between two N levels, within a *Lupinus* genotype.
5 - LSD (0.05), between two growth stages within a *Lupinus* genotype.
6 - LSD (0.05), between *Lupinus* genotypes, averaged across N levels and growth stages.

Table 6. Effects of nitrate levels and growth stages on C_2H_2 reduction activity of six *Lupinus mutabilis*: two cultivars as control (cvs. '176', 'SCG-25'), two hypernodulating (L-114, L-105) and two low-nodulating mutants (L-123, L-57)[1]

		Reduction activity (μmoles $C_2H_2.h^{-1}.plant^{-1}$)[2]							
		cv.'176'	cv.' SCG-25'	L-114	L-105	L-123	L-57	Mean	LSD[3]
N	0mM	3.14	2.76	5.07	1.59	1.33	1.97	2.64	0.28
level	5mM	0.88	0.58	1.10	0.80	0.53	0.34	0.71	
	LSD[4]	0.68							
Growth	7 weeks	1.93	1.70	3.42	2.16	1.33	1.68	2.04	0.28
Stage	10 weeks	2.09	1.65	2.76	0.23	0.54	0.64	1.31	
	LSD[5]	0.68							
	Overall Mean	2.01	1.67	3.09	1.19	0.93	1.15		
	LSD[6]	0.97							

1 - Hypernodulating and low nodulating mutants are designated according to a previous selection work done in 1987 and 1988.
2 - Each value is an average of 12 plants contained in four Leonard jars with 3 plants. jar^{-1}.
3 - LSD (0.05), between N levels and growth stages, averaged across *Lupinus* genotypes.
4 - LSD (0.05), between two N levels, within a *Lupinus* genotype.
5 - LSD (0.05), between two growth stages within a *Lupinus* genotype.
6 - LSD (0.05), between *Lupinus* genotypes, averaged across N levels and growth stages.

Table 7. Effects of nitrate levels and growth stages on specific nodule activity of six *Lupinus mutabilis*: two cultivars as control (cvs. '176', 'SCG-25'), two hypernodulating (L-114, L-105) and two low-nodulating mutants (L-123, L-57)[1]

		Specific reduction activity (μmoles $C_2H_2.h^{-1}.plant^{-1}$)[2]							
		cv.'176'	cv.' SCG-25'	L-114	L-105	L-123	L-57	Mean	LSD[3]
N	0mM	4.23	4.35	8.84	4.24	3.71	3.09	4.74	1.52
level	5mM	2.83	2.83	4.39	2.89	2.28	1.64	2.81	
	LSD[4]	1.14							
Growth	7 weeks	5.20	5.73	10.58	6.82	5.12	4.27	6.28	1.14
Stage	10 weeks	1.86	1.44	2.65	0.31	0.87	0.46	1.27	
	LSD[5]	1.14							
	Overall Mean	3.53	3.59	6.62	3.56	2.99	2.36		
	LSD[6]	1.61							

1 - Hypernodulating and low nodulating mutants are designated according to a previous selection work done in 1987 and 1988.
2 - Each value is an average of 12 plants contained in four Leonard jars with 3 plants. jar^{-1}.
3 - LSD (0.05), between N levels and growth stages, averaged across *Lupinus* genotypes.
4 - LSD (0.05), between two N levels, within a *Lupinus* genotype.
5 - LSD (0.05), between two growth stages within a *Lupinus* genotype.
6 - LSD (0.05), between *Lupinus* genotypes, averaged across N levels and growth stages.

increased demand by the effective nodules of the mutants may be the cause of this phenomenon (17). According to Wu and Harper (25), the addition of nitrate to well nodulated soybean plants resulted in a decreased nitrogenase activity in all three selected nodulation mutants and in the cultivar Williams, which is a wild type. However, the nitrogenase activity detected in the mutants was much less affected than that in cv. Williams.

Gremaud and Harper (13) reported that nitrate was less inhibitory to infection and initial nodule development in the soybean mutant lines than in the cv. Williams. It has also been observed by ^{15}N analysis that the three selected soybean mutants considered as nitrate tolerant symbionts are more capable of symbiotically fixing nitrogen than their parents when grown with nitrate (18). Moreover, in the studies conducted by Gremaud and Harper (13) and Ohyama and Harper (18), the authors could not indicate whether nitrate was diminishing the activity of the nitrogenase system. The overall pattern of declining C_2H_2 reduction activity reported by Wu and Harper (25) illustrates the short-term adverse effect of nitrate on nitrogenase activity.

The enzymatic activities of N assimilation (glutamine synthetase) and the carbohydrate availability for the N-fixation metabolism (cytosol PEP-carboxylase) are shown in Table 8. The results demonstrate a similar pattern between nitrogenase activity and glutamine synthetase. The GS activity in mutants L-114 and 105 showed a higher performance than in the cv. 'SCG-25' under NO_3^- addition, but the cv. '176' had a marked and significant increase in GS with nitrate application, while the other genotypes

Table 8. Effect of nitrate on glutamine synthethase (GS) and phosphoenolpyruvate carboxylase (PEPC) activities in the nodules cytosol of 7 weeks old plants of *Lupinus mutabilis*[1]

Genotype	N-level	Activity	
		GS (μmoles GH.h^{-1}.protein mg^{-1})	PEPC (μmoles PEPC.h^{-1}.protein mg^{-1})
cv. '176'	0mM	0.64	15.50
	5mM	1.82	93.34
cv. 'SCG-25'	0mM	1.28	11.90
	5mM	0.77	29.78
L-114	0mM	1.44	25.53
	5mM	1.32	80.13
L-105	0mM	1.72	35.00
	5mM	0.75	71.15
L-123	0mM	0.80	52.08
	5mM	0.65	27.52
L-57	0mM	2.20	17.27
	5mM	1.16	21.92
LSD[2]		0.81	41.41

[1] - Hypernodulating (L-114, L-105) and low nodulating mutants (L-123, L-57) are designated according to a previous selection work done in 1987 and 1988.
[2] - LSD (0.05) between N levels and *Lupinus* genotypes.

presented a decrease in such activity. Table 8 also shows a high increase of the phosphoenolpyruvate carboxylase activity in all cultivars and mutant lines supplemented with nitrogen, with the exception of L-123.

There were no differences in GS activity of nodules between *Lupinus* mutants L-114 and L-105 and cv. 'SCG-25' when the plants were grown without nitrate. However, in the presence of nitrate, there was a trend for the mutants, mainly L-114, to show higher GS activity. This indicates that L-114 is more capable of assimilating N that is symbiotically fixed and has a higher carbohydrate supply as indicated by the PEP-carboxylase activity. There were significant differences in PEP-carboxylase activity between mutants L-114 and L-105 and their parents in both the presence and absence of nitrate. The difference in PEP-carboxylase activity among genotypes reflects the effect of nitrate treatment, also associated with a marked effect on nodule mass and nitrogenase activity. Thus, the partial tolerance to nitrate presented by L-114 and L-105 was apparently related to the hypernodulated phenotype. These results can be seen in Tables 6 and 7, where the acetylene reduction activities per plant and per gram of nodules were higher for mutant L-114 than for cv. 'SCG-25', both in the absence and presence of nitrate.

From these results it is concluded that the *Lupinus* mutants L-114 and L-105 were more efficient with respect to nodulation, N-yield, and nitrogenase activity than the wild type cv. 'SCG-25' and may allow higher nitrogen fixation in the presence of nitrate in the nutrient solution. The five millimolar concentration of nitrate surrounding the roots appeared to be most important for the inhibition of initial nodulation, although the data show that the mutants had partial tolerance to nitrate and predict some gain in overall nitrogen metabolism with a greater ability to assimilate fixed N and higher amounts of available carbohydrates. However, the current study was not able to distinguish whether this effect was due to a mutation in the nodulation process or, to some extent, to a change in N metabolism.

RESUMO

Efeitos do nitrato na nodulação e fixação de N_2 em mutantes de *Lupinus mutabilis* ao longo do ciclo de crescimento

Uma das principais técnicas usadas para estudar o efeito inibidor do NO_3^- na fixação biológica do N_2, tem sido o uso de mutantes de estirpes de *Rhizobium* ou de plantas hospedeiras que não apresentam o padrão

usual de nodulação. Este estudo foi conduzido usando genótipos parentais e mutantes de *Lupinus mutabilis*, submetidos a dois níveis de NO_3^- : 0 e 5mM, com duas épocas de colheita: sete e dez semanas após emergência. A concentração de 5mM revelou-se da maior importância na inibição da nodulação inicial de *Lupinus*, apesar de os dados demonstrarem que os mutantes L-114 e L-105 foram mais eficientes quanto a nodulação, N-total e atividade da nitrogenase que a cv."SCG-25", e podem permitir um aumento da fixação de N_2. Mas não houve diferenças na atividade da glutamina sintetase (GS) dos nódulos entre os mutantes L-114, L-105 e a cv. "SCG-25", quando as plantas não receberam NO_3^-, mas na presença de NO_3^- houve tendência para os mutantes, especialmente L-114, apresentarem maior atividade da GS. Isto indica que o mutante L-114 tem uma maior capacidade de assimilação do N fixado, associada com um maior suprimento de carboidratos disponíveis, como indicado pela atividade da PEP-carboxilase. Portanto, a tolerância parcial ao NO_3^- apresentada pelos mutantes L-114 e L-105 está associada com o fenótipo hipernodulante. Esta sugestão pode ser observada nos resultados da atividade total e específica de redução de acetileno, que foram maiores para o mutante L-114 que para a cv. "SCG-25", na presença ou na ausência de NO_3^-.

Palavra-chave: *Bradyrhizobium*, glutamina sintetase, phosphoenolpiruvate carboxilase, citosol nodular, metabolismo do N, redução de acetileno.

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THE ROLE OF THE EXTERNAL MYCELIAL NETWORK OF ARBUSCULAR MYCORRHIZAL (AM) FUNGI. II. A STUDY OF PHOSPHORUS TRANSFER BETWEEN PLANTS INTERCONNECTED BY A COMMON MYCELIUM

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ABSTRACT

The transfer of ^{32}P from *Lolium perenne* (the donor) to *Plantago lanceolata* (the receiver) mediated by arbuscular mycorrhizal (AM) fungi was examined when the two species were grown together or separately. The arbuscular mycorrhizal infection led to a significant increase in the amount of ^{32}P transferred from donor to receiver plants relative to that in uninfected plants, not only when the roots of the two plants were growing in intimate mixture but also when they were separated by a root-free zone of 2.33 cm. The majority of isotope transfer between the two plant species occurred by a direct pathway via AM mycelium.

Key words: Arbuscular mycorrhizal fungi, ^{32}P transfer, soil microbiology.

INTRODUCTION

It has been documented, by the use of isotope-tracer methods, that colonization of plant roots by arbuscular mycorrhizal (AM) fungi can provide channels for the transfer of ^{32}P between associated plants (7, 8, 9, 12, 13, 15). Two major mechanisms could be involved in this process: (a) direct transfer of phosphorus between plants through the mycelium which connects individuals, a process that may be enhanced by the generally low levels of host specificity shown by AM fungi; (b) indirect transfer involving leakage of phosphorus from roots of one plant, its absorption by AM mycorrhizal hyphae scavenging in the rhizosphere, and its transfer to neighboring plants. In addition to these two, there is a third possible mechanism which is not mediated by AM fungi. This involves the leakage of phosphorus from roots of one plant and its subsequent absorption by the roots of neighboring plants. However, the extent to which these mechanisms are involved in the transfer process of P between plants is not very well understood because the experiments designed to study them have not so far provided the information necessary to discriminate between them.

In an attempt to provide a better understanding of the mechanisms mediated by AM fungi of phosphorus transfer between plants, the present work used the carrier-free isotope ^{32}P and chambers specifically designed to facilitate discrimination between direct and indirect ^{32}P transfer. The extent to which simulated grazing could lead to a stimulation in the rate of ^{32}P transfer between plants was also examined.

MATERIALS AND METHODS

Square pots (7x7x12 cm) were constructed using a plastic drain pipe. Each pot was divided longitudinally

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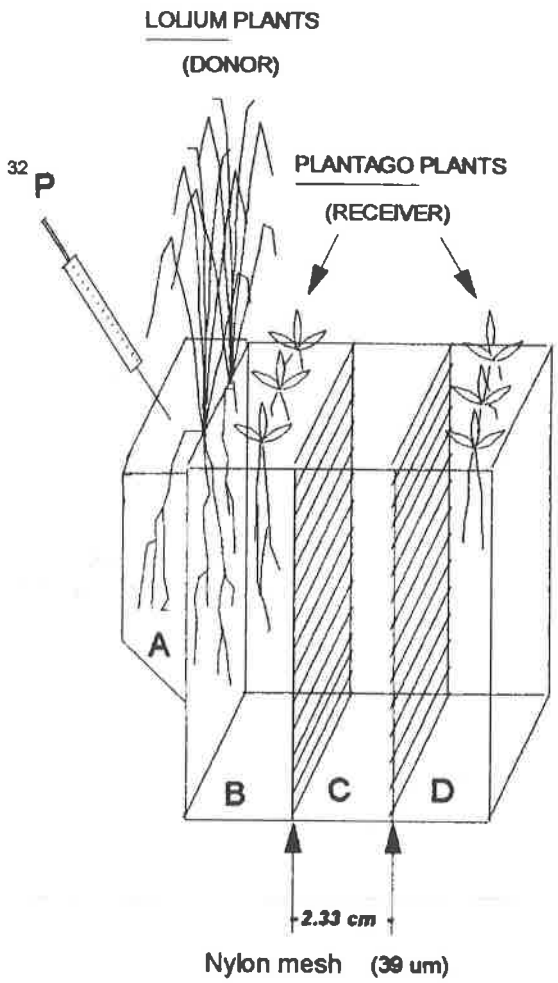

Figure 1. Design of the constructed pots, showing the feeding of ^{32}P. (not in scale).

into three identical sections (160 cm^3) by two pieces of nylon mesh screen (Nybolt PA-40/23; 39-mm mesh screen and 23% of free surface). The nylon acted as a barrier to plant roots but allowed AM hyphae to pass freely. Four 10-cm vertical cuts were made into the walls of each pot starting at the base, leaving a 2-cm uncut section at the top. The nylon screens were then fed through theses slits and glued to the outside of the pot. Plastic bases were then attached to the bottom of the pots. A further section of approximately 150 cm^3 capacity was also glued to one of the longitudinal faces of the pots. The final configuration of the pots is depicted in Fig. 1.

Each pot section was filled with twice autoclaved dune sand and either two pre-infected mycorrhizal (M) or two non-mycorrhizal (NM) control plants of *Lolium perenne* were carefully arranged with their roots divided, so that approximately half of their root systems were growing in each of sections A and B (Fig. 1). The mycorrhizal *Lolium* plants were produced by growing them for 3 weeks in small trays (21x16x5 cm) containing approximately 30g of root segments infected with *Glomus etunicatum* and spread to form two layers of inoculum between two layers of autoclaved dune sand. In the case of the NM control plants, approximately 25 mL of filtrate solution (filter paper Whatman N°. 1) derived from root washings was added to the sand, in order to ensure that the control plants received a bacterial population comparable with that present in the mycorrhizal medium yet lacking AM propagules.

The pots were placed in a controlled-environment growth room with an 18h light period at 20-25°C and 6h dark period at 15-18°C. The pots were watered every 2 days with distilled water. After 35 days, 5 mL of a nutrient solution containing N=20 ppm, P=8 ppm, K=50 ppm, Ca=30 ppm, Mg=10 ppm, Fe=1 ppm, Mn=0.1 ppm, B=0.2 ppm, Mo=0.04 ppm, Zn=0.04 ppm, and Cu=0.04 ppm, were added to each section of the pots. Ten weeks after the *Lolium* plants had been planted, 3 pre-germinated seeds of *Plantago lanceolata* were planted into sections B and D (FIG. 1). The *Plantago* plants in section D were thus isolated from those in B by the whole of section C, a distance of approximately 2.33 cm, while those in section B were growing with their roots in intimate mixture with half of the divided root system of the *Lolium* donor plants.

Thirty-one days after transferring the *Plantago* specimens the *Lolium* donor plants were fed with ^{32}P by injecting into the feeding chamber (A) 1.8 MBq of ^{32}P as orthophosphate in 40 mL of water using a Hamilton microsyringe.

Four days after feeding, two treatments were imposed:

1. In half of the M (M -Lol shoots) and half of the NM (NM -Lol shoots) pots, the *Lolium* shoots were removed by cutting them at ground level. Any regrowth of the clipped plants was further removed. This treatment was designed to simulate grazing.
2. In the remaining half of the M (M +Lol shoots) and NM (NM +Lol shoots) pots, the shoots of *Lolium* donor plants were allowed to develop normally.

There were 10 pots for each treatment, five with M and five with NM plants.

Eighteen days after the donor roots had been fed, the plants in each compartment were individually harvested and freed from sand particles. One of the receiver *Plantago* plants in each section was used to determine the percentage of root length infected with AM fungi (4). The remaining plants were used for determination of the levels of radioactivity.

For determination of the levels of radioactivity, plant material was oven-dried (48 h at 80°C), weighed and ground. Thereafter, sub-samples of roots or shoots of known dry weight were placed in vials and digested by adding 10 mL of the one-step scintillant/digestant Fluorosol. The vials were incubated for 24 h at 50°C to ensure complete digestion of the plant material and subsequently kept in a cool, dark place for 24 h to permit subsidence of any chemiluminescence. The levels of radioactivity were measured using a liquid scintillation counter (Packard, Tri-Carb 300C and 300CD). All counts were corrected for color quenching, background and isotope decay.

RESULTS

The levels of AM colonization in the roots of *Plantago* receiver plants were not affected either by the defoliation of the donors or by their position in relation to the donor plants (Table 1). The presence of infection confirmed that AM mycelium had grown from donor to receiver plants, forming AM links even when the plants were separated by two barriers and 2.33 cm of sand.

The amounts of ^{32}P transferred to M *Plantago* receivers from *Lolium* donor plants were always significantly higher than in their NM counterparts in both treatments (Table 2). The concentration of ^{32}P measured in the receivers was, as could be expected, higher when the two species had their root systems intermingled (section B) than when the species were separated (section D). In the M plants, however, concentrations of ^{32}P in roots of receivers in both +Lol and -Lol treatments reached values approximately 1/10 than those in the B compartment. If *Lolium* donor plants were maintained intact, virtually no activity was transferred to non-mycorrhizal receivers in section D.

Removal of donor shoots led to a greater than 100-fold increase of activity in shoots and roots of M receivers in both sections B and D, relative to that seen in the +Lol shoot treatment. In contrast, the NM receivers showed a very small increase in counts in shoots and roots in the D compartment, these still having levels of radioactivity that were scarcely above background (Table 2).

Expressed as a percentage of the total activity added to section A of each pot, the M receivers in the -Lol shoot treatment growing in B and D

Table 1. Percentage of root length infected by AM fungi in the *Plantago* receiver plants.

AM STATUS	TREATMENT			
	+L shoots		-L shoots	
	B	D	B	D
M	72	74	74	75
NM	0	0	0	0

N=5
+L shoots= *Lolium* shoots were preserved intact;
-L shoots= *Lolium* shoots removed 4 days after ^{32}P feeding;
B= Donor and receiver plants growing with their root systems intermingled (section B);
D= Receiver roots separated from the donor roots by two nylon mesh screens (39 µm) with a gap of 2.33 cm between them (section D).

Table 2. Concentrations of ^{32}P in tissue samples of *Plantago* receiver plants eighteen days after part of the roots of *Lolium* donor plants had been fed with ^{32}P.

TREATMENT	VA STATUS	^{32}P RADIOACTIVITY (dpm/mg dry wt.)					
		SHOOT		ROOT		SHOOT + ROOT a	
		B	D	B	D	B	D
	M	826	21	975	97	1,801 a	118 a
	NM	21	0	145	2	226 b	2 b
	N	15,669	649	11,989	1,373	27,658 c	2, 019 c
	NM	75	16	98	36	173 b	52 d

N=5
+L shoot= *Lolium* shoots preserved intact;
-L shoot= *Lolium* shoots removed 4 days after ^{32}P feeding;
B= Donor and receiver plants growing with their root systems intermingled (section B);
D= Receiver roots separated from the donor roots by two nylon mesh screens (39 µm) with a gap of 2.33 cm between them (section D);
dpm= disintegrations per minute;
[a] values followed by the same letter, in the same column, are not statistically different (P=0.05) by *t*-test.

Table 3. Total radioactivity and percentage of that which was added into the A compartment of each pot (10.8×10^7 dpm) in the whole *Plantago* receiver plants, eighteen days after ^{32}P feeding.

TREATMENT	AM STATUS	32P RADIOACTIVITY (dpm) *PLANTAGO* a		RADIOACTIVITY IN RECEIVER AS % OF THAT ADDED TO THE POTS	
		B	D	B	D
+L	M	57,722 a	10,318 a	0.05	0.001
	NM	5,689 b	74 b	0.005	0.00007
-L	M	718,789 c	186,587 c	0.7	0.2
	NM	11,651 d	7,182 a	0.01	0.006

N=5
+L= *Lolium* shoots preserved intact;
-L= *Lolium* shoots removed 4 days after ^{32}P feeding;
B= Donor and receiver plants growing with their root systems intermingled (section B);
D= Receiver roots separated from the donor roots by two nylon mesh screens (39 µm) with a gap of 2.33 cm between them (section D);
dpm= disintegrations per minute;
[a] Values followed by the same letter, in the same column, are not statistically different (P=0.05) by *t*-test.

compartments gained 0.7 and 0.2% respectively of the isotope after 18 days, while NM plants growing in these positions and M and NM plants in the +Lol shoot treatments gained negligible proportions of the isotope (Table 3).

DISCUSSION

The presence of infection in the receiver plants confirmed that AM fungi had grown from donor to receiver roots, even when they were separated by a 2.33 cm zone of sand from which roots were excluded. Bethlenfalvay *et al.* (1) and Camel *et al.* (2) have shown that AM hyphae can spread from their associated roots into the soil over distances of 6 and 9 cm. Since the onset of sporulation of AM fungi requires 4-8 weeks and new spores have an endogenous dormancy cycle of 6 weeks to 6 months (14), the colonization observed in receiver roots is probably exclusively due to growth of mycelium from roots of donor plants. Read *et al.* (11) observed that most plants in semi-natural grassland become heavily infected very soon after seed germination. They suggested that infection of the developing root system must arise from contact with mycelium spreading from plants with established infection, and that as a consequence of this pattern of infection many plants within the community must be inter-linked by mycorrhizal hyphae. This has since been confirmed by studies of the development of infection of seedlings in the field (10).

The results showed that AM infection led to significant increases in the quantities of ^{32}P transferred from donor to receiver plants relative to that seen in uninfected plants, both when the roots of the two species were growing in intimate mixture and also when they were separated by a root-free zone of 2.33 cm. In the case of NM plants, transfer of isotope occurred from donor to receive plants growing with their roots intermingled but only negligible quantities were transferred across the two nylon mesh barriers. This confirms that a direct pathway involving transfer of P through mycelial inter-connections is occurring. The importance of this pathway is increased more than an order of magnitude following defoliation (simulating grazing) of the donor plants. Evans (3) showed that removing some of the foliage from *Lolium perenne* (simulating grazing) reduces root growth, and if the defoliation is severe and repeated some roots die, although the plant as a whole remains alive.

The amounts of isotope transferred from NM donors to NM receivers in the "B" compartment in both conditions between intact living plants and also when the roots of donor started to die following defoliation (simulating grazing) suggests that a pathway exist. Results from other workers have also demonstrated that such a pathway exist between intact living plants (8, 9, 15) and from dying roots to living plants (7, 13). It is also necessary to account for the very large amount of ^{32}P transferred from M donor to M receiver plants when their roots were closely intermingled in the "B" compartment. There are a number of possible bases for this transfer. One of them is that the transfer is by the indirect mechanism involving leakage from donor and re-absorption from the soil pool by receiver roots. However, examination of ^{32}P transfer from donor to receiver in the NM conditions does not suggest that such a pathway is of predominant importance, unless the unlikely assumption is made that mycorrhizal infection

increases the tendency of donor plant to "leak" P. In fact, Newman and Ritz (9) showed that the loss of ^{32}P from *Lolium* roots to the solution during a 22-day period was not influenced by mycorrhizal infection. Hence, it must be concluded that the greater amount of ^{32}P transfer between donor and receiver in M plants in section B (root systems closely intermingled) relative to that in section D (root systems separated) is due either to a greater number of hyphal bridges in the densely intermingled root systems, or to the fact that this short path length will facilitate increased rates of P flux through the hyphae. Furthermore, the external AM hyphae of infected receiver plants will certainly increase the effectiveness of scavenging for any eventually ^{32}P leaked from the donor roots. In reality it is most likely that a combination of the two first effects is responsible for the large ^{32}P transfer seen in M receiver in section B (direct transfer pathway through the hyphae). Newman and Ritz (9) assumed that there would be a greater total amount of nutrient transferred by direct hyphal links than via the soil pool model. Also, Whittingham and Read (15) concluded that direct transfer of nutrients between living source and sink plants occurs mainly by way of connecting mycorrhizal hyphae. However, Ikram *et al.* (6) found no significant transfer of P from *Pueraria phaseoloides* to *Hevea brasiliensis* via hyphal links of AM fungi.

The proportion of ^{32}P amounts which reached the M receiver plants in "B" and "D" compartment (0.7 and 0.2%, respectively) following defoliation of the donors appears to be small relative to that added to the "A" compartment, but if such amounts are being transferred to very small receivers every 18 days, they may be large enough to be considered of potential ecological importance. It is clear that these significant proportion of ^{32}P that reached the receivers when the donor shoots were removed is entirely mediated by the presence of fungi, since the amount of ^{32}P transferred to NM receiver plants was not different in either whether donor shoots had been removed or not. The better efficiency of the fungi in leading a greater isotope transfer following defoliation of the donors could be simply because a facilitated release of ^{32}P occurs when the donor roots start to die. This could be stimulated by the presence of the fungi due the formation of a P concentration gradient generated when P was being translocated through the hyphae to sink receiver roots, which, in turn, may be controlled by the demand of the receiver plants. Heap and Newman (5) measured ^{32}P in the shoots of *Plantago* receiver plants 7 days after the *Lolium* donor plant had been detopped and found that 2-3% of the ^{32}P in the donor roots were transferred to shoots of *Plantago* if the plants were M. Later, Ritz and Newman (13) showed that most of the transfer from dying *Lolium* roots occurred in a surge, during the second to third week after removal of the donor shoot. They found, in the mycorrhizal pots, that of the ^{32}P present in *Lolium* roots at the time of removal 31% was transferred to the *Plantago* shoot within a 22-day period. Hence, it seems quite possible that, when roots are dying, ^{32}P can pass more efficiently from host to fungus, the reverse of the normal direction, thus allowing direct transfer to occur.

The results revealed that AM fungi can provide channels for direct transfer of P between individual plants. The importance of this pathway was greatly intensified by removing the shoots of the donor plants (simulating grazing). The possible significance of such transfer process for individuals and for communities of plants is that it can facilitate effective recycling of P while restricting the potential for its chemical and physical fixation or immobilization by saprophytic microorganisms in the soil.

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RESUMO

Significância do micélio externo dos fungos micorrízicos arbusculares. II. Estudo da transferência de fósforo entre plantas inter-conectadas por um mesmo micélio

A transferência de ^{32}P entre *Lolium perenne* (planta doadora) e *Plantago lanceolata* (planta receptora), mediada por fungos micorrízicos arbusculares (FMA), foi avaliada quando as espécies cresceram juntas ou separadas. Os resultados demonstraram que a infecção micorrízica arbuscular proporcionou aumento significativo na quantidade de ^{32}P transferida das plantas doadoras para as receptoras, não somente quando as mesmas foram cultivadas com os sistemas radiculares juntos, mas também quando tiveram seus sistemas radiculares separados por uma distância de 2,33 cm. A maior quantidade de ^{32}P

transferido entre as duas espécies de plantas ocorreu através da transferência direta via micélio fúngico que as interligou.

Palavras-chave: Fungos micorrízicos arbusculares, transferência de P, microbiologia do solo.

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MOLECULAR EPIDEMIOLOGY OF *HAEMOPHILUS INFLUENZAE* TYPE B ISOLATES FROM URUGUAY

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ABSTRACT

Little information is available about the molecular epidemiology and genetic relationships of *Haemophilus Influenzae* serotype b (Hib) recovered from patients living in South America. Forty-three Hib isolates cultured largely from patients with invasive diseases in Uruguay between 1987 and 1992 were characterized for multilocus enzyme genotype and outer membrane protein (OMP) subtype. OMP analysis identified three of the known subtypes, including 3L (70%), 2L (18%), and 18L (2%). Two previously undescribed patterns related to the L and U subtype families were also found, and each represented 5% of the organisms. Four multilocus enzyme electrophoretic types were identified in the sample, including ET 12.5 (67.3%), 12.8 (23.3%), 12.7 (4.7%), and an ET closely related to ET 25.6 (4.7%). The combination of OMP subtype and ET allowed the recognition of 7 distinct Hib subclones. The ET 12.5/3L subclone accounted for 63% of all isolates, and caused 83% of the meningitis cases but only 55% of the episodes of pneumonia and sepsis. In general, the frequency of occurrence of Hib subclones in Uruguay was similar to that recorded in several European countries.

Key words: *H. influenzae* b, enzyme genotype, outer membrane proteins.

INTRODUCTION

Haemophilus influenzae serotype b (Hib) is a major cause of bacterial meningitis and other serious infections in Uruguayan children. The organism is the second most common cause of childhood meningitis in Uruguay, and is surpassed only by disease due to *Neisseria meningitidis* in epidemic years. Hib is also the second most frequent cause of bacterial pneumonia in children less than 5 years old in Uruguay (4). Currently, the Hib vaccine is not used in this country. Because Hib is a significant cause of pediatric morbidity, it is especially important to understand the molecular epidemiology of diseases caused by the organism.

With the exception of limited data available from studies of serotype b *H.influenzae* strains recovered in restricted settings in Brazil and Chile (10), there is little known about the nature and extent of diversity of strains cultured from patients in South America. Study of *H. influenzae* strains recovered from serious infections in Uruguayan children has shown that virtually all invasive episodes are caused by organisms expressing serotype b capsule, and most are biotype I (5). However, Hib strains from Uruguay have not yet been analyzed by commonly employed epidemiological techniques such as outer membrane protein subtyping (2) and multilocus enzyme electrophoresis (9). The primary goal of this study was to subtype a sample of Hib strains recovered in Uruguay in order to gain insight into the molecular epidemiology of strains causing pediatric infections in this country. A secondary goal was to determine the extent of subclone infection type associations.

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The Country. Uruguay is a small South American country (176,215 km2), with a temperate climate (6). Almost one-half of its 3 million inhabitants live in the capital city of Montevideo. The ethnic background of the population is largely European. Blacks and Amerindians together only represent 3%.

Bacterial isolates. Between 1987 and 1992, 37 Hib were isolated from blood, pleural fluid, cerebrospinal fluid or other normally sterile body site. Six additional strains were recovered from the upper respiratory tract of patients with severe acute respiratory infections.

MATERIALS AND METHODS

Identification of the strains was made by colony morphology, Gram stain, and growth requirements(1). Isolates were serotyped by counterimmunoelectrophoresis (CIE) with anti-Hib serum generously provided by Dr. D.M.Granoff, Washington University School of Medicine, or SSI, Denmark, and by coagglutination (CoA) (Phadebact, Karo Blo Diagn., AB Sweden).

The Hib isolates were previously tested for urease and ornithine decarboxylase activity and indole production (7). All the strains were assigned to biotype I, except one isolate from acute otitis media which was biotype II, and one invasive strain identified as biotype IV. Strains were stored at -70°C in skim milk.

OMP profiling. A procedure for rapid preparation of detergent-insoluble outer membrane proteins was used (3). Briefly, 4 to 6 colonies of a fresh culture were inoculated in 10 ml of brain-heart infusion broth supplemented with 2% Fildes and incubated overnight on a rotary-shaker incubator (Precision Scientific 360) at 120 rpm. Cells were harvested by centrifugation (5,000 x g) for 10 min. at 4°C. The pellet was resuspended in 1.5 ml of 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethansulfonic acid) buffer (pH 7.4) and stored at -70°C, or immediately sonicated on ice (6 impulses of 10 minutes each, at 50 w, in ultrasonic homogenizer), cellular debris were removed by centrifugation (5,000 x g) for 2 min. at 4°C. The supernatant was centrifuged again, and the cell envelopes were suspended in 0.2 ml of HEPES and incubated at room temperature for 30 min. in an equal volume of 2% sodium lauryl sarcosinate. Centrifugation was repeated and the OMP preparation was resuspended in 100 µl of HEPES and stored frozen.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein content in OMP samples was determined by a modified Lowry method. The samples were then diluted to 1 mg/ml of protein in a buffer containing 0.5 TRIS (pH 6.8) 10% glycerol, 2% SDS and 5% mercaptoethanol, and heated at 100°C for 5 min. These OMP preparations were analyzed in a 15% polyacrylamide gel with molecular weight standards (range 18,5-106 KD, Blo-Rad Laboratories, Richmond, CA). Known OMP sub types (2, 12) (provided by L.van Alphen, University of Amsterdam, The Netherlands) were included as controls. The gels were stained with Coomassie blue and OMP patterns were determined by comparison with the reference strains. The OMP nomenclature described by Barenkamp *et al.* (2) was used.

Multilocus enzyme electrophoresis. Allellic variation at 12 metabolic enzyme loci was assayed by multilocus enzyme electrophoresis (9). Briefly, isolates were grown in brain heart infusion broth supplement with 2% Fildes in a shaker incubator bath at 35°C for 18 hrs. Cells were harvested by centrifugation, resuspended in 1.5 ml 50 mM TRIS, pH 7.5, containing 5 mM EDTA and sonicated for 30 seconds with ice-water cooling. After centrifugation at 14,000 x g for 20 minutes at 4°C, the lysates were stored at -70°C.

Horizontal starch gel electrophoresis was performed on the lysates, and specific enzyme activities were assayed (9). The following 12 enzymes were analyzed for electrophoretic variation: phosphoglucose isomerase (PGI), malic enzyme (ME), malate dehydrogenase (MDH), glucose 6-phosphate dehydrogenase (G6P), 6-phosphogluconate dehydrogenase (6PG), glutamic oxaloacetic transaminase (GOT), adenylate kinase (ADK), two peptidases (PEP1 and PEP2), phosphoglucomutase (PGM) nucleoside phosphorylase (NSP) and carbamylate kinase (CAK).

Variation in the electrophoretic mobility of the enzymes was determined visually by comparing their relative migrations side by side on the gels. The allele designations described previously (9) were used. Electrophorectic types (ETs) were assigned according to the combination of electromorphs of each enzyme over all loci (9).

Table 1. Allelic profiles of electrophoretic types (ET) detected in Uruguay

			Investigated enzymes*											
ETs	n	%	CAK	NSP	PGI	ME	MDH	G6P	GOT	ADK	6PG	PEP2	PEP1	PGM
12.5	29	67.4	5	5	4	5	5	3	2	2	2	4	5	4
12.8	10	23.2	8	5	4	5	5	3	3	2	2	4	5	4
12.7	2	4.7	7	5	4	5	5	3	3	2	2	4	5	4
99+	2	4.7	6	5	5	3	3	6	3	2	2	4	4**	6

* For abbreviations see methods
** PEP1 migration different from 25.6
\+ New ET

RESULTS

Multilocus enzyme electrophoretic analysis of the 43 isolates identified 4 distinct ETs. ET 12.5 was the most common (67.3%). ET 12.8 was detected in 23.3% of the strains and ET 12.7 in 4.7%. The other two strains, provisionally designated as "new", differed from ET 25.6 at the peptidase 1 locus (Table 1). All 4 multilocus enzyme types (clones) were represented among the invasive strains. Interestingly, ET 12.5 caused 83% of meningitis cases, but only 55% of the pneumonia episodes.

OMP analysis of the 43 Hib identified 5 distinct profiles (OMP subtypes) (Fig. 1). OMP subtype 3L accounted for 70% of cases, followed by OMP 2L (18%), OMP 18L (2%) and undetermined U and L OMP subtypes (5% each). Of the 37 isolates cultured from normally sterile sites, 27 were OMP 3L and 7 were assigned to subtype 2L.

The 4 ETs and 5 OMP profiles together identified 7 distinct combinations, marking subclones. ET 12.5/OMP 3L was the most prevalent subclone and accounted for 63% of all isolates. With a single exception, all ET 12.5/OMP 3L organisms were biotype I. This subclone comprised 55% of the invasive pneumonia isolates, and 83% of the strains recovered from cerebrospinal fluid. A miscellaneous group formed by isolates cultured from acute otitis media, cellulitis, abscess and throat strains had a similar frequency to that of this subclone (55%) (Table 2). Eight Hib isolates assigned to subclone 12.8/2L were evenly distributed among invasive and non-invasive strains. Two other subclones were represented by 2 isolates each: ET 12.7/OMP 3L and ET 12.5/30L. Another 2 groups were only detected in one strain each (12.8/18L and 12.8/3L). In addition, the strains related to ET 25.6 had a "U" OMP, but the entire OMP pattern was not identical to any of the reference Hib isolates (6U, 12U from Barenkamp (2); type 3 from van Alphen (12).)

Study of the age distribution of the patients infected with above mentioned subclones indicated that 12.5/3L and 12.8/2L both caused disease in infants, but only the 12.5/3L subclone occurred in children older than 12 months.

Table 2. Clonal Distribution By Anatomic sites of Hib isolation

ET / OMP	CSF n/%	Blood/Pleural fluid n/%	Miscellaneous* n/%	Total n/%
12.5/3L	10/83.3	11/55	6/55	27/62.8
12.8/2L	2/16.7	4/20	2/18	8/18.6
12.7/3L	-	1/5	1/9	2/4.65
12.8/3L	-	1/5	-	1/2.3
99/20U++	-	2/10	-	2/4.65
12.5/30L++	-	1/5	1/9	2/4.65
12.8/18L	-	-	1/9	1/2.3
Total	12/27.9	20/46.5	11/25.6	43/100

* Throat or middle ear exudates, abscess, cellulitis.
++ Arbitrary numbers given to undescribed ET and OMPs.

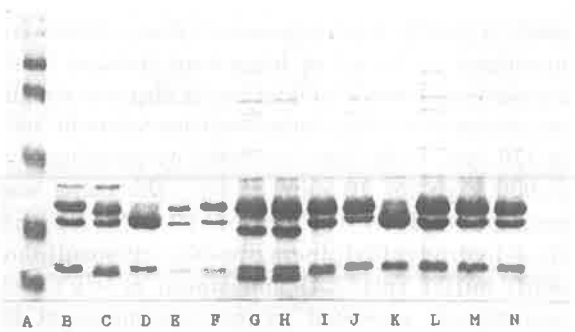

Figure 1. Outer membrane protein (OMP) electrophoretic profiles of representative strains from Uruguay. OMP electrophoretic profiles of Hib strains were generated by a rapid microprocedure described elsewhere (3). Strains of known OMP subtypes were included as controls. Lane A, molecular weight markers; B reference subtype (RS) 12U; C, Uruguayan strains (US), U; D, RS 2L; E, RS IL; F, RS 3L; G, RS 18 L; H, US 18L; I, US 3L; J, US L (undesignated); K, US 2L; L-N, US 3L.

Temporal variation in the frequency of occurrence of the 7 subclones was also detected. For example, in 1987-88, subclone 12.5/3L was the most abundant, but in the following years this subclone had an almost equal distribution compared with the other subclones.

DISCUSSION

Analysis of several thousand serotype b *H. influenzae* isolates recovered from patients living in North America, Europe, Africa, Asia and elsewhere has found that nine clones account for 81% of cases of invasive disease worldwide (9,10.). Most cases of Hib invasive disease in the United States are caused by isolates of two clones marked by ET 1.9/OMP 1H and ET 12/OMP 3L. A small proportion of disease (<5%) is attributable to the ET 25.6/OMP 6U clone. In striking contrast to the situation in the United States, most disease episodes in northern and western Europe are caused by strains of ET 12.5 OMP 3L (type 1 in the van Alphen system). With the exception of limited data generated from epidemiologic analysis of organisms in Chile (8) and Brazil, (10) there are no significant insights into the molecular population structure of strains recovered from patients in South America. In as much as future immunoprophylaxis strategies may depend in part on knowledge of local strain subtypes, especially OMP subtypes, we thought it important to investigate clonal relationships among a larger sample of strains from Uruguay drawn from patients with no known epidemiological relationship.

Our data demonstrate that in Uruguay most disease episodes are caused by Hib strains assigned to ET 12.5/OMP 3L, and ET 12.8/OMP 2L subclones. In general, the subclone distribution in Uruguay was similar to that described for European countries studied (9,10).

Although significant geographic variation in the frequency of occurrence of Hib clones and subclones occurs, with the exception of a single report from Finland, (11) there is little evidence to suggest biologically meaningful differences in relative pathogenicity among the commonly occurring Hib subclones . In Finland, Takala *et al.* (11) reported that strains of OMP subtype 1c, which are usually ET 21.8, caused proportionately more meningitis and less epiglottitis than strains of OMP subtype 1, which are usually assigned to ET 12.5. In our study, ET 12.5/OMP 3L strains caused 83% of the meningitis cases, but only 55% of pneumonia episodes. However, this trend should be interpreted cautiously because of the relatively small Hib sample size.

In abstract, virtually all Hib strains recovered from diseased children in Uruguay can be assigned to two distinct subclones. The data are the first comprehensive assessment of the distribution of Hib subclones from a country in South America. Additional studies should be conducted to examine the nature and extent of genetic variation present in other countries in South America, especially those with substantially greater native population.

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RESUMO

Epidemiologia molecular de *Haemophilus influenzae* tipo b no Uruguai

A informação disponível sobre a epidemilogia molecular e as interrelações genéticas dos *Haemophilus influenzae* serotipo b (Hib) recuperados de pacientes que vivem na América do Sul é escassa. Quarenta e três cepas isoladas de pacientes com doença invasiva no Uruguai entre 1987 e 1992 foram caracterizadas por genótipo enzimático multilocus e por subtipo de proteína de membrana externa (outer membrane protein: OMP). As análises de OMP identificaram três dos subtipos conhecidos, incluindo 3L (70%), 2L (18%) e 18L(2%).

Também se encontrou dois padrões, não descritos anteriormente, relacionados às famílias dos subtipos L e U, cada um representando 5% dos organismos. Quatro tipos electroforéticos de enzimas multilocus foram identificados, incluindo ET 12.5 (67.3%), 12.8 (23.3%), 12.7 (4.7%), e um ET intimamente relacionado ao ET 25.6 (4.7%). A combinação de subtipo OMP com ET permitiu o reconhecimento de 7 suclones Hib diferentes. O subclone ET 12.5/3L foi responsável por 63% de todos os isolamentos, e causou 83% dos casos de meningite mas somente 55% dos episódios de pneumonia e septicemia. Em geral, a freqüência da ocorrência de subclones Hib no Uruguai foi igual à registrada em vários países da Europa.

Palavras-chave: *H. influenzae* b, genótipo enzimático, proteínas de membrana externa.

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PENICILLIN-BINDING PROTEINS OF PATHOGENIC *YERSINIA* SPECIES

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ABSTRACT

The penicillin-binding proteins (PBPs) of *Yersinia pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* grown at 28°C or 37°C were detected after labeling with [^{3}H]-benzylpenicillin and fluorography of polyacrylamide gels. Each sample showed a unique PBP profile composed of three to six proteins, with molecular weights ranging from 120,000 to 43,000. Incubation at 37°C resulted in significant changes of the PBP profiles of all three species. The possible implications of these results on the physiology of these bacteria and on the action of β-lactam antibiotics are discussed.

Key words: penicillin-binding proteins, PBPs, *Yersinia*, cell wall.

INTRODUCTION

The β-lactam antibiotics, like the penicillins and the cephalosporins, kill bacteria by inhibiting cross-linking of murein strands of the bacterial peptidoglycan, the cell wall (15, 18). The targets of these antibiotics are enzymes anchored to the outer face of the cytoplasmic membrane by short hydrophobic carboxy or amino terminal sequences. These enzymes are found in all peptidoglycan-containing bacteria, usually in numbers of 2 to 6 and with molecular weights ranging from 120 to 40 kDa. The β-lactam-sensitive enzymes are commonly detected by their ability to bind penicillin covalently, hence the name penicillin-binding proteins (PBPs) (6, 9,13-16, 18).

Based on the stability of the complex PBP-penicillin, Spratt (14) developed a simple electrophoretic procedure to detect them in gels. Incubation of whole cells or isolated envelopes with radioactive penicillin followed by sorting in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography have permitted the rather easy detection of PBPs. Although the number, size and relative concentration of individual PBPs vary widely among different species, taxonomically related bacteria appear to share similar PBP patterns (6, 16). In spite of the availability of a simple PBP detection procedure, the PBP content of some important human pathogens like those belonging to the *Yersinia* genus has not been investigated.

The *Yersinia* genus includes three species pathogenic to humans: *Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis*. The last two are associated with gastroenteritis in humans and are transmitted by the oral/fecal route. The primary infection site of these organisms is the lymphoid follicle of the small intestine, causing enteric infections with symptoms that range from mild diarrhea to severe systemic infection in a few cases (2). *Y. pestis*, the causative agent of plague, is a highly invasive pathogen transmitted by flea bites. The plague bacillus shows a marked tropism for lymph nodes, where it multiplies mainly at extracellular sites, giving rise to the buboes from where it spreads to other organs and leads to a fatal septicemic process (2). The three *Yersinia* species

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share similar anti-host defense mechanisms which result in evasion of phagocytic clearance. Several anti-phagocytic proteins are encoded by the 65-75 kb virulence-associated plasmid which is highly conserved among the three species (1, 2, 9, 11). The *in vitro* expression of virulence-associated proteins, the Yop proteins, requires incubation at 37°C in low calcium containing media (5, 17). The same environmental conditions lead also to growth restriction, nutritional requirements and altered cell morphology (17). Regulation of this complex process requires chromosomal and plasmidial genes and is called the low calcium response (LCR) (2, 5, 17).

In this work the PBPs of the three pathogenic *Yersinia* species were detected and their numbers and sizes determined. In addition, a characteristic change in the PBP profile of the each species was observed after raising the temperature to 37°C. The possible consequences of temperature-dependent PBP changes in these organisms are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The three *Yersinia* strains used in this work were: *Y. pestis* EV76 (10), *Y. enterocolitica* 8081 (12) and *Y. pseudotuberculosis* YPIII (1). Organisms were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 28°C or at 37°C on a New Brunswick rotating water bath shaker. Frozen stocks, prepared in BHI with 25% glycerol and maintained at -70°C, were used to inoculate cultures containing 25 ml of BHI in 125 ml Erlenmeyer flasks which were next incubated at 28°C for approximately 24 hours. The cultures were then diluted 1:10 (*Y. pestis*) or 1:100 (*Y. enterocolitica* and *Y. pseudotuberculosis*) in 250 ml of BHI and were grown for one hour at 28°C before the shift to 37°C. Cells were harvested 36 (*Y. pestis*) or 16 hours (*Y. pseudotuberculosis* and *Y. enterocolitica*) after the temperature shift.

Preparation of membranes. The cells were harvested by centrifugation (7,000 x g for 10 min) at 4°C, washed once with 50 mM sodium phosphate buffer (pH 7.0), resuspended in 10 ml of phosphate buffer and subjected to sonic disruption with cooling. After removal of intact cells, the cell envelope fraction was recovered by centrifugation of the sonic extracts at 100,000 x g for 30 min. Pellets were washed once, carefully solubilized in phosphate buffer with the aid of a glass rod and vortexing, and stored at -20°C.

Detection of PBPs by labeling with [^{3}H]-benzylpenicillin. Aliquots of cell envelopes with approximately 100 µg total protein were labeled with 10 µg/ml of [^{3}H]-benzylpenicillin (21 C/mmol; Amersham, Little Chalfont, United Kingdon) in a total volume of 20 µl during 20 min at 30°C. Labeling was interrupted by addition of 2 mg benzylpenicillin in phosphate buffer. After centrifugation (12,000 rpm in a microcentrifuge at 4°C), the pellets were solubilized with 20 µl of a 3% Sarkosyl solution (Sigma, St. Louis, U.S.A.) and kept at room temperature for 20 min. Samples were centrifuged once more in a microcentrifuge set at 12,000 rpm in the cold for 20 min. The supernatants were transferred to another tube containing 10 µl of 2 x concentrated electrophoresis sample buffer and boiled for 3 minutes. Labeling of *Y. enterocolitica* PBPs required the previous inactivation of endogenous β-lactamase with 1 mg/ml of clavulanic acid.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were fractionated by SDS-PAGE (T= 10%, C = 1.66% running gel and T= 5%, C=2.8% stacking gel) according to Lammeli (1970). The electrophoresis apparatus used throughout was the Mini Protean II system from Bio-Rad Laboratories (Richmond, Calif., U.S.A.) and the runs were performed at 100V for approximately 90 minutes. For fluorography, gels were equilibrated with 1 M sodium salicylate containing 0.5% glycerol for 30 min, rinsed briefly and dried under vacuum over a sheet of 3MM filter paper in a gel dryer apparatus set at 60°C. Dried gels were placed against X-ray films (Kodak XAR-5) and kept at -70°C for 7 days. When necessary autoradiograms were analyzed in a Quick Scan Jr. densitometer (Helena Laboratories, Beaumont, Tex.) coupled to a Varian 4290 integrator (Varian, Walnut Creek, Calif., U.S.A.). Radioactive molecular weight markers were purchased from Gibco (Gibco Laboratories, Gaithesburg, MD, U.S.A.). The proteins and their molecular weights were: myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

Protein determination. Protein content was determined according to Lowry *et al.* (8) with bovine serum albumine as the standard.

RESULTS

The number of PBPs found in the envelopes of the three species was dependent on the species analyzed and the incubation temperature. The total number of bands observed in fluorograms ranged from 3 (in *Y. enterocolitica* grown at 37°C) to 6 (in *Y. pestis* at 28°C and *Y. pseudotuberculosis* at both temperatures) and molecular weights ranged from 43,000 to 120,000 (Fig. 1 and Table 1). PBPs were predominantly found in the sarkosyl-soluble fraction of the bacterial cell envelopes.

Several differences in the PBP profiles of the *Yersinia* species were observed after growth at the higher temperature. The most dramatic case was that of *Y. pestis* EV76, which showed a gradual reduction of all PBPs except PBP2 after the shift to 37°C. Incubation of *Y. pestis* at 37°C for 36 hours resulted in a PBP profile composed of PBP2 and residual amounts of PBP4, 5 and 6 (Fig. 1). When *Y. enterocolitica* cells were incubated at 37°C two new prominent PBPs with molecular weights of 78,000 and 45,000 were detected. Moreover, the mobility of PBP1 was slightly reduced after overnight incubation of *Y. enterocolitica* at the elevated temperature (Fig. 1 and Table 1). The PBP profile of *Y. pseudotuberculosis* showed several changes after overnight incubation at 37°C, like a reduction in the levels of PBP1, 3 and 4 and induction of two PBPs with molecular weights of 92,000 (PBP2') and 78,000 (PBP3'), not present in cells grown at 28°C (Fig. 1 and Table 1). PBPs 5 and 6 were not altered by the temperature shift.

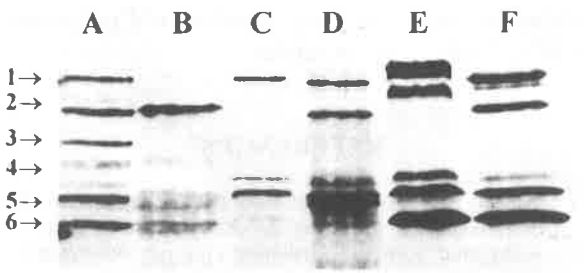

FIGURE 1. Fluorogram of a SDS gel of solubilized membranes of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* after labeling with [^{3}H]-benzylpenicillin. The membranes were isolated from *Y. pestis* EV76 (A and B); *Y. enterocolitica* 8081 (C and D) and *Y. pseudotuberculosis* PIII (E and F) grown at 28C (A,C and E) or 37°C (B,D and F). The nomenclature of *Y. pestis* PBPs grown at 28°C follows that previously described by Ferreira *et al.* (1994) and is indicated on the left side of the figure.

DISCUSSION

In this report we describe a unique PBP composition, both in number and size, for each of the three pathogenic *Yersinia* species investigated, namely: *Y. enterocolitica*, *Y. pestis* and *Y. pseudotuberculosis*. These patterns differed from those found in other Gram-negative bacteria like *Escherichia coli*, but similarities between the profiles of *Y. pestis* and *Y. pseudotuberculosis* were evident. The PBP profiles obtained allowed the distinction

Table 1: The PBPs of *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*.

Organism	Incubation temperature (°C)	PBP - Mr (x 10^3)
Y. pestis EV76	28	1 - 97 2 - 78 3 - 61 4 - 56 4* - 48 [a] 5 - 46 6 - 43
	37	1 - - 2 - 78 3 - - 4 - 56 [b] 5 - 46 [b] 6 - 43 [b]
Y. enterocolitica	28	1 - 97 2 - - 3 - 54 4 - 47 5 - -
	37	1 - 95 2 - 78 [c] 3 - 54 4 - 47 5 - 45
Y. pseudotuberculosis	28	1 -120 2 - 98 3 - 85 4 - 55 5 - 47 6 - 43
Y. pseudotuberculosis	37	1 - - 2 - 98 2* - 92 [a] 3 - - 3* - 78 [c] 4 - 55 [b] 5 - 47 6 - 43

a - minor PBP representing less than 5 % of the total PBPs, not detected in some gels.
b - PBP reduced at 37°C to less than 25% of the amounts observed in samples incubated at 28°C.
c - new PBP detected only in samples incubated at 37°C.

between the three *Yersinia* species and might represent a potential diagnostic tool. In a previous work we demonstrated that the PBP profiles of several *Y. pestis* strains grown at 28°C were similar (4). However, the screening of a larger number of strains, including wild type isolates, should be carried out before any relevant taxonomic value could be attributed to the *Yersinia* PBP profiles.

All three *Yersinia* species showed modifications of their PBP patterns upon incubation at 37°C yet the physiological significance of this process is unknown. Modification of the PBP pattern at 37°C in *Y. pestis* is dependent on the induction of the low calcium response (LCR) encoded by the virulence-associated plasmid (3). In line with other LCR-regulated phenotypes in *Yersinia*, addition of millimolar concentrations of Ca^{2+} to the growth medium inhibits the changes of PBP profiles induced by the shift to 37°C (3; and unpublished observations). These evidences suggest that the stability and/activity of the *Yersinia* PBPs might share a control mechanism with the virulence-associated LCR regulon. Similarly, the observed changes in PBP profiles under LCR-inducing conditions indicate that the peptidoglycan metabolism is altered in these cells and might be relevant to the pathogenicity of *Yersinia*.

The high molecular weight PBPs (in the range of 100,000 to 60,000) represent essential enzymes involved in transpeptidation and transglycosylation activities of the cell metabolism (Park, 1987; Spratt, 1975; 1983). The changes in high molecular weight PBPs observed in the *Yersinia* species incubated at 37°C seem to alter the availability of lethal targets to some β-lactam antibiotics. Evaluation of the *in vitro* susceptibility of *Y. pestis* to β-lactams at 37°C is not feasible due to its poor growth at elevated temperature under LCR-inducing conditions, whereas *Y. enterocolitica* produces β-lactamases which prevent the detection of PBP-associated resistance to these antibiotics. Nonetheless, *Y.pseudotuberculosis* did show significantly higher MICs *in vitro* to some β-lactams at 37°C (data not shown). Although incubation at elevated temperatures could alter the permeability of the cell envelope by other mechanisms, the temperature-dependent modification of the PBP profiles may be an additional factor for the usual failure of β-lactam-based therapy in *Yersinia* infections.

In a previous work we showed that the high molecular PBPs 2 and 3 of *Y. pestis* have physiological roles similar to those of their counterparts in *Escherichia coli* K12, that is, control of cell shape and septum formation, respectively (4). Definition of the physiological roles of the *Yersinia* PBPs and understanding of the mechanism leading to changes in the PBP profile under LCR-inducing conditions may contribute to the development of new generation β-lactam antibiotics with high affinity for essential PBPs present in the cell envelope of *Yersinia* grown under different conditions.

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RESUMO

As proteínas ligadoras de penicilina (PBPs) das espécies patogênicas de *Yersinia*

As PBPs de *Yersinia pestis*, *Y. enterocolitica* e *Y. pseudotuberculosis* crescidas a 28°C ou a 37°C foram detectadas após marcação com [^{3}H]-benzilpenicilina e fluorografia dos géis de poliacrilamida. Cada amostra apresentou um perfil único de PBPs composto por três a seis proteínas com peso molecular variando entre 120.000 e 43.000. Incubação a 37°C resultou em mudanças significativas nos perfis de PBPs das três espécies estudadas. As possíveis implicações destes resultados na ação dos antibióticos β-lactâmicos e na fisiologia destas bactérias são discutidas.

Palavras-chave: proteínas ligadoras de penicilina, PBPs, *Yersinia*, parede celular.

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ACINETOBACTER SPECIES IN CLINICAL SPECIMENS: BIOTYPES AND SEROTYPES OF *ACINETOBACTER BAUMANNII* STRAINS ISOLATED IN SÃO PAULO, BRAZIL

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ABSTRACT

Hundred and ninety *Acinetobacter* strains (76 from cerebrospinal fluid, 30 from blood and 84 from other clinical specimens) isolated during the period 1986-92, in São Paulo, Brazil, were identified according to Bouvet and Grimont and Bouvet and Jeanjean.The serotypes and biotypes were determined for *Acinetobacter baumannii* strains, following the methods described by Traub, and Bouvet and Grimont, respectively. 92.6% of the isolates were classified into 10 species and *A.baumannii* (60.0%), and *A.genospecie* 3 (11.6%) were the most frequent ones. Biotypes 2, 6, 9, and serotypes 04, 015 and 029 were predominant among *A.baumannii* strains. Besides *A.baumannii*, other species as *A.genospecies* 3 and *A.lwoffi*, were mainly isolated from septicemia and meningitis cases, which show the significance of these species also as important opportunistic pathogens.

Key words: *Acinetobacter*; *Acinetobacter baumannii*; biotypes, serotypes

INTRODUCTION

Members of the genus *Acinetobacter* have the water and soil as their main habitat, and can also be found as a normal flora of skin in 17-20% of healthy individuals (2, 4, 31).

This opportunistic pathogen is rarely a cause of community-acquired infections. Nevertheless, in the last decades, *Acinetobacter* species have been recognized as an increasingly serious problem in nosocomial infections (5, 17, 26, 27, 37).

Hospital-acquired infections by *Acinetobacter* seem to be associated with hospital instrumentation and the use of contaminated devices, and the hands of hospital personnels contribute to the dissemination of these infections (1, 3, 11, 30).

Infections in debilitated patients, usually under antibiotic therapy or with severe underlying diseases, result in septicemia, meningitis, endocarditis, brain and lung abscess as well as other clinical manifestations (26, 27, 30).

A striking feature which is also of great concern is the appearence of multiply-resistant strains reported by several authors which implies on continuous surveillance on drug resistance and evaluation of new antibiotics to be used in therapy with satisfactory results (6, 20, 21, 35, 36).

The taxonomy of the genus *Acinetobacter*, which remained confused for several decades, changed substantially with the study of Bouvet and Grimont(7). By DNA/DNA hybridization techniques they delineated 12 genospecies, 11 of which could be identified by phenotypical characteristics.

Currently, by DNA/DNA hybridization, 17 genomic groups have been reported in the genus *Acinetobacter*, and *A. baumannii* represents the

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species most frequently isolated from clinical specimens (7, 8, 9, 10, 24, 28).

Although in the past many typing methods had been applied to differentiate *Acinetobacter* strains, they rendered useless because of the taxonomic changing. Recently, methods of biotyping, serotyping, plasmid profile analysis as well as other molecular biology techniques were developed to discriminate isolates most frequently associated with human infections (8, 12, 13, 15, 18, 19, 29, 32).

Given the frequent isolation of members of genus *Acinetobacter* from clinical specimens, our purpose was to characterize its species and to determine the biotypes and serotypes of *A.baumannii* because of its significance in hospital environment.

MATERIAL AND METHODS

Bacterial strains. A sample of hundred ninety *Acinetobacter* strains isolated during the period 1986-92 (76 from cerebrospinal fluid, 30 from blood, and 84 from other clinical specimens) were included in this study. The majority of strains had been isolated in two hospitals, in São Paulo, Brazil. Reference strains of genomic groups used as control were supplied by Dr P.J.M. Bouvet and Dr P.A.D. Grimont from Service des Entérobactéries, Institut Pasteur, Paris, France. *Acinetobacter baumannii* type strains representative of 28 serogroups were kindly provided by Dr W.Traub, Institut für Mikrobiologie und Hygiene, Federal Republic of Germany. Strains 345/91 and 774/91, corresponding to the new serogroups 029 and 030, were used as reference strains for sera production (34).

Identification of the strains. All strains, sent as non-fermentative gram negative rods, were identified as *Acinetobacter* by the characteristics described in Bergey's Manual of Systematic Bacteriology (23) and confirmed by Juni's transformation assay (22). Species identification was performed according to Bouvet and Grimont (7) and Bouvet and Jeanjean (9), using the following tests: growth at 37°, 41°C and 44°C, production of acid from glucose, gelatin hydrolysis, hemolysis on blood (sheep) agar and carbon source utilization (DL-lactate, glutarate, L-phenylalanine, phenylacetate, malonate, L-aspartate, L-leucine, histamine, L-tyrosine, β-alanine, azelate, D-malate, 2,3 butanediol, trans-aconitate, DL-4-aminobutirate, trigonelline, L-ornithine, tryptamine, protocatechuate, quinate, benzoate, 4-hydroxybenzoate, gentisate).

Biotyping of *A.baumannii*. The biotypes of *A. baumannii* were determined according to Bouvet and Grimont (8) based on carbon source utilization from the substrates: levulinate, citraconate, L-phenylalanine, phenylacetate, 4-hydroxybenzoate and L-tartrate.

Serotyping of *A.baumannii*. Antisera were produced according to Traub (32). Isolates were subjected to tube agglutination test and performed as described by Traub (32, 33), except that the antigenic suspensions were adjusted to 1 x 10^8 cells / ml. Isolates were previously tested against 30 antisera diluted 1/20, and each strain was agglutinated against serially twofold diluted antiserum (positive at previous test) starting from 1/10 dilution.

RESULTS

92.6% of the isolates were classified into 10 genospecies: *A.baumannii* (60.0%), *A.*genospecie 3 (11.6%), *A.lwoffi* (7.9%), *A. haemolyticus* (5.3%), *A.*genospecie 12 (2.1%), *A.johnsonii* (1.6%), *A.*genospecie 13 (1.6%), *A.junii* (1.0%), *A.*genospeceie 10 (1.0%) and *A.*genospecie 14 (0.5%). The distribution of *Acinetobacter* species according to the source of isolation is shown in Fig. 1.

Among 110 *A. baumannii* isolates, 10 biotypes were delineated with the predominance of biotypes 9 (37.3%), 2 (28.2%), and 6 (19.1%). In the Table 1 is shown the distribution of the biotypes following the source of the isolation.

The serotyping method allowed the identification of 87.7% of *A.baumannii* strains, and the most frequent serotypes were 04 (17.0%), 029 (17.0%) and 015 (11.3%) among 19 disclosed serotypes.

In Table 2 is shown the association between serotypes and biotypes. All the isolates belonging to serotype 029 were associated with biotype 2, and 94.4% of serotype 04 were biotype 9.

DISCUSSION

The taxonomic revision of the genus *Acinetobacter*, undertaken by Bouvet and Grimont (7) based on DNA/DNA hybridization method confirmed how wide and heterogeneous the group of this microorganism is. Their study was essential for the

Table 1. Distribution of the biotypes of 110 *A.baumannii* strains according to the source of isolation

	biotypes										Total
	1	2	5	6	8	9	10	11	12	13	
source of isolation											
CSF		15		6	1	7	2	2			33
blood		2	1	3		9					15
secretions	1	8		8		15	3			1	36
urine	2	3		3		4	1		1		14
tip catheter		2		1		2	1		1		7
unknown human origin		1				3				1	5
Total	3	31	1	21	1	40	7	2	2	2	110

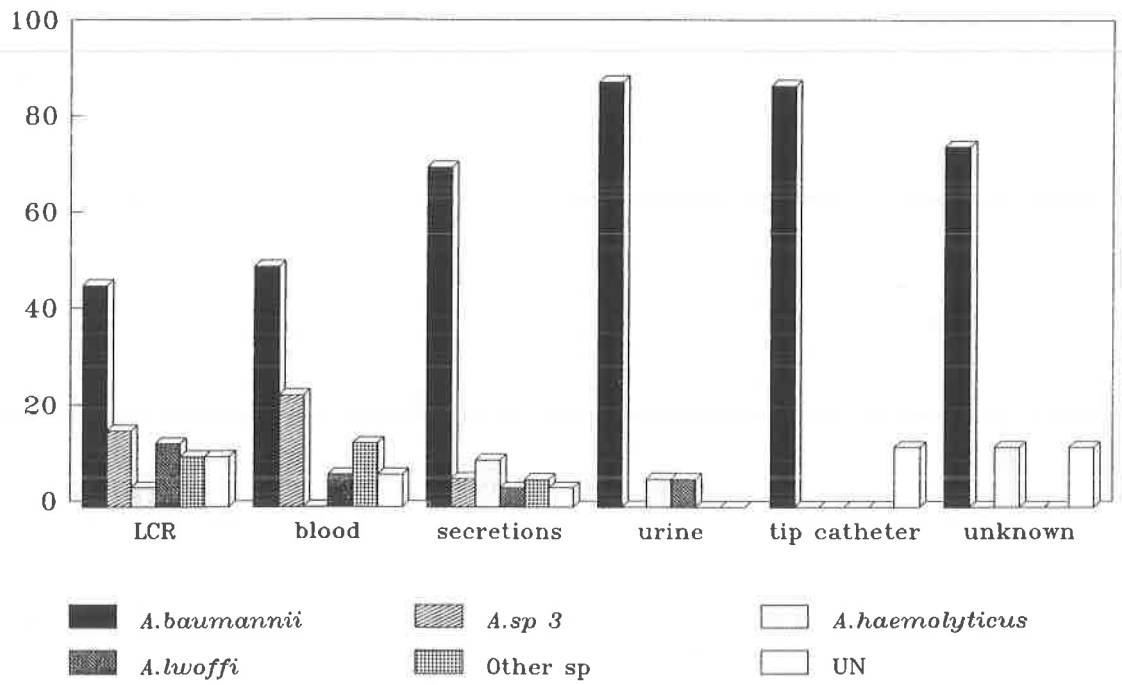

Figure 1. Percentage of *Acinetobacter* species according to the sources of isolation

advance of taxonomy of the genus, and the phenotypical characteristics established by them is an useful reference system to recognize species in clinical microbiology laboratories.

The increasing reports concerning the role of *Acinetobacter* in hospital-acquired infections with *A. baumannii* as the most frequently associated species among 17 delineated genospecies, emphasizes the need of an accurate identification of clinical isolates to study the ecology and the epidemiology of this microorganism.

In Brazil, although rarely mentioned as nosocomial pathogen, it should have an important role as confirmed by the data obtained from this study.

Consistent with the data of other investigators (14, 24), a high percentage (92.6%) of the samples could be identified based on Bouvet and Grimont (7) method, and *A. baumannii* has been found as a

prevalent species in clinical specimens.The presence of this species in hospital environment, and as a possible nosocomial infection cause is shown by its recovery from CSF and blood cultures (Fig. 2).

Among several proposed typing methods for differentiation of *A.baumannii* strains (8, 15, 18, 25, 32), we assayed biotyping and serotyping which revealed useful to discriminate the isolates, and both should be used as an important epidemiological marker to delineate nosocomial outbreaks.

The biotypes 9, 6, and 2 were prevalent among *A.baumannii* strains, and the recovery of the biotype 9 (60.0%) and biotype 2 (45.4%) from blood and CSF cultures, respectively, highlights an important role of these strains in hospital-acquired infections. According to Bouvet and Grimont (8), it seems that a given biotype is preferentially associated with the site of isolation.

Interestingly, the recently described (34) serotype 029, and serotype 04 predominated among isolates. According to Traub (32, 33) and Oliveira (24) the serotypes 04, 029, and 013 were the most frequent among their samples. A striking feature was the association between the serotype 029 with biotype 2 and of 04 with biotype 9 (Table 2).

Besides the *A.baumannii*, other species as *A.genospecie* 3 and *A.lwoffi*, were mainly isolated from septicemia and meningitis cases, which show the significance of these species also as important opportunistic pathogens.

It should be emphasized that strains belonging to genospecies 13 and genospecies 14 were identified following the method described by Bouvet and Jeanjean (9), which correspond to their proteolytic group. Because of the controversial taxonomic position of the genomic groups 1, 2, 3 and 13, suggested by Gerner-Smidt *et al.*(16) to be named as complex *A.calcoaceticus-baumannii*, further study is needed in order to carry out a full characterization of these species, taking into account their significance in nosocomial infections.

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Table 2. Number of associations between serotypes and biotypes of 105 *A. baumannii* isolates

	biotypes										Total
	1	2	5	6	8	9	10	11	12	13	
serotypes											
02						2	1				3
04		1				17					18
07					1	1	1				3
09		1		1							2
010			1	2			1				4
011		3					1				4
012						1					1
013		1		5		1					7
015	1	1		3		3		2	1	1	12
016				1		1					2
018						1					1
019				1							1
020		1				1	1				3
022				2							2
024							1				1
025				1		1			1		3
028				1		1					2
029		18									18
030		4				3					7
NT*	2	1		2		5	1				11
Total	3	31	1	19	1	38	7	2	2	1	105

* non typable with 30 tested antisera

Institut für Medizinische Mikrobiologie und Hygiene, Universität des Saarlandes, Federal Republic of Germany for supplying type strains.

RESUMO

Espécies de *Acinetobacter* em materiais clínicos: biotipos e sorotipos de cepas de *Acinetobacter baumanii* isoladas em São Paulo, Brasil

Foram estudadas 190 cepas de *Acinetobacter* (76 isoladas de líquido cefalorraquidiano, 30 de sangue e 84 de outros materiais clínicos), isoladas no período 1986-92, em São Paulo, Brasil. As espécies foram identificadas segundo Bouvet and Grimont e Bouvet and Jeanjean. Os sorotipos e biotipos foram determinados para *A.baumannii* seguindo os métodos descritos por Traub e Bouvet and Grimont, respectivamente. Entre as 10 espécies identificadas, o *A.baumannii* (60%) e *A.genospecies* 3 (11.6%) foram as mais freqüentes. Verificou-se que os biotipos 2, 6, e 9 e os sorotipos 04, 015 e 029 foram predominantes entre as cepas de *A.baumannii*.

Palavras-chave: *Acinetobacter*, *A.baumannii*, biotipos, sorotipos.

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STAPHYLOCOCCUS AUREUS AND *SALMONELLA ENTERITIDIS* PRESENT IN FOOD IMPLICATED IN FOOD POISONING

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ABSTRACT

Two hundred-eighty people became ill with diarrhea, vomiting, fever, cramps, and headache 6 to 15 hours after consuming potato salad at a restaurant in Brasilia, Brazil, on September 3, 1993. Both *Staphylococcus aureus* (2.1 x 10^6/g) and *Salmonella enteritidis* were isolated from the potato salad, the only food remaining from the dinner. Because other food poisoning agents were not present in the potato salad and other foods consumed at the dinner were not likely suspects for food poisoning because of estimated low attack rates, it was concluded that the illnesses could have been due to the staphylococci and *Salmonella* contaminating the potato salad. The symptoms overlap those of salmonellosis and staphylococcal food poisoning, except for fever which seldom occurs in the former. The time limit is within the range for salmonellosis, with 6 hours normally being the longer time limit for staphylococcal food poisoning. The staphylococci may have come from the food handlers and the *S. enteritidis* from the eggs used in preparing the potato salad dressing. Salmonellosis from eggs have occurred in other countries, with *S. enteritidis* being present inside the eggs. The staphylococci were not examined for the production of enterotoxin nor was the food examined for the presence of enterotoxin. On examination, several of the food handlers in the restaurant were colonized with enterotoxigenic staphylococci. This is important information because most staphylococcal food poisoning outbreaks result from contamination of the food by the food handlers.

Key words: Staphylococci, *Salmonella enteritidis*, food poisoning, enterotoxin.

INTRODUCTION

Food poisoning can be caused by a number of different organisms, with the leading cause being *Salmonella*. This type of food poisoning can result in serious illnesses with death resulting in some cases. The major symptom is diarrhea, with vomiting, intestinal pain, fever, and headache as accompanying symptoms usually in 12-36 hours, but can be in the range of 5-72 hours following contact with *Salmonella* (5), depending on the number of *Salmonella* present in the food. The onset of symptoms is delayed because it is necessary for the organisms to grow in the intestinal tract to result in illness. Normally the symptoms for each type of food

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poisoning is unique and can be distinguished from the symptoms associated with other foodborne disease agents. For example, the symptoms for staphylococcal food poisoning are nausea, vomiting, abdominal cramping, and diarrhea, within 1 to 6 hours (1). The symptoms are of short duration, a few hours to one day, whereas the symptoms of salmonellosis may last for several days. Seldom is staphylococcal food poisoning fatal, whereas death from salmonellosis is not unusual. Staphylococcal food poisoning is the result of toxins being produced in the food by the organisms growing there, to at least 10^6/g of food (10, 12). In contrast, salmonellosis can result from relatively few infecting microorganisms in the food because disease requires invasion of the intestinal tract and toxin production.

Contamination of the food occurs by different routes. Animals are a very important reservoir of *Salmonella*, although the bacterial pathogen can be found in other ecological niches as well (5). Staphylococci, however, are commonly associated with humans, a common source of food contamination (2). Adequate cooking of the food will destroy both *Salmonella* and staphylococci, but the food often is recontaminated with staphylococci after the cooking by food handlers.

This communication describes a food poisoning outbreak in which the food was contaminated with both staphylococci and *Salmonella*. The possible sources of the contaminations are discussed.

MATERIALS AND METHODS

The outbreak. Two-hundred-eighty individuals (70%) of 400 who dined at a restaurant in Brasilia on September 3, 1993 became ill 6 to 15 hours, with a median of 9 hours, after eating. Of the 39 individuals interviewed, all (100%) had diarrhea, 30 (75%) had vomiting, with several experiencing stomach cramps and headache. There were no deaths and all recovered within two or three days. All had consumed potato salad, which contained potatoes, corn cream, eggs, peas, and salad dressing made with whole shell eggs. The attack rate for the potato salad was 81.8%. The attack rate for the other foods, rice, beans, breast of chicken, gnocchi, and beef roll, was only estimated.

Examination of the foods. The potato salad and 12 whole shell eggs were examined for the presence of *Salmonella* as follows (6). Twenty-five grams of the salad dressing was homogenized in 225 ml of buffered peptone water and incubated for 24 hours at 37°C. One milliliter of the pre-enrichment was added to 10 ml of tetrathionate with brilliant green broth and bile salts; 1 ml was added to 10 ml of Rappaport-Vassiliadis and incubated for 24 hours at 37°C. The enrichments were streaked on brilliant green agar and SS agar and incubated for 24 hours at 37°C. One colony from each medium was transferred to triple sugar agar and to lysine iron agar and incubated for 24 hours at 37°C. Serological testing with somatic and flagella antibodies was performed to determine the *Salmonella* species.

Isolation of staphylococci. Twenty-five grams of the potato salad was suspended in 225 ml of buffered peptone water; 0.1 ml was placed on Baird-Parker agar plates and incubated for 48 hours at 37°C (9). A typical colony was transferred to two test tubes containing 1 ml of BHI broth and incubated for 24 hours at 37°C. Tests for coagulase and thermonuclease (TNase) production, anaerobic fermentation of glucose and mannitol, and production of hemolysin using sheep blood were made. Any colonies that were positive for these characteristics were considered *S. aureus*. For staphylococcal count, additional Baird-Parker plates were prepared with 10-fold dilutions if necessary (9). The typical colonies that showed these characteristics were counted and the number multiplied by the dilution factor and recorded as cfu/g of food.

The food handler's nares were cultured using sterile swabs each week for four weeks. The same procedures were used for isolation of the staphylococci from the nasal swabs.

Enterotoxin production. For enterotoxin production, inocula were prepared by combining five isolates from each culturing and incubating them in brain heart infusion (BHI) broth over night at 37°C. Enterotoxin production was by the membrane-over-agar plate method described by Robbins *et al.* (11).

Enterotoxin testing. The optimum-sensitivity-plate (OSP) gel diffusion method was used as described by Robbins *et al.*(11). The more sensitive RPLA method also was used for testing the culture supernatant fluids for enterotoxin (8). The RPLA method (Oxoid) employs latex particles coated with the specific antisera to the enterotoxins, each on separate latex particles. In the presence of enterotoxin, the latex particles agglutinate. They are sensitive to about 0.5 ng of enterotoxin.

RESULTS

Detection of *Salmonella*. S. enteritidis was detected in the potato salad from the incriminated meal. No other foods from the actual meal were available for examination. None of the eggs examined were positive for *S. enteritidis*.

Isolation of staphylococci from the food. *S. aureus* was isolated from the potato salad at a count of 2.1×10^6 cfu/g. Also, *S. aureus* was isolated from 7 of 14 food handlers.

Production of enterotoxin. The staphylococci isolated from the potato salad was not examined for the production of enterotoxin because at the time of the outbreak this was not possible and the staphylococci were not retained. The staphylococci isolated from 4 of the 7 food handlers produced enterotoxin (Table 1).

DISCUSSION

It is uncommon for two types of food poisoning agents to be involved simultaneously in an outbreak. In this outbreak, both *Salmonella* and *S. aureus* were isolated in sufficient numbers from the potato salad, the only leftover food, to be involved. Other food poisoning organisms, such as *Bacillus cereus* and sulfite reducing clostridia were not present. Unfortunately, no patient's stools were examined for *Salmonella,* However, *Salmonella* could have been the major contributor to the symptoms observed. Much smaller numbers of *Salmonella* are required to produce illness than for staphylococci. Although 10^6 cfu/g of staphylococci were present, this is the lower limit for the production of adequate amounts of enterotoxin to cause illness (10, 12). It is possible that the initial illnesses at six hours was due to staphylococcal enterotoxin followed later by salmonellosis.

The *Salmonella* were present in the potato salad and likely came from the eggs used in preparing the salad dressing. Although the extra eggs examined were not contaminated with *Salmonella*, usually only a small percentage (<1%) of eggs are internally contaminated (7). Only one contaminated egg is required and in the present case a few *Salmonella* in a contaminated egg could have increased as the potato salad, initially refrigerated for a short time, was held at room temperature (30°C) for over five hours, before serving. This length of time was adequate for the staphylococcal count to increase to 10^6 cfu/g (10,12), from the presumptively low numbers initially introduced by an infected food handler. Other ingredients of the potato salad, such as the potatoes, corn, and peas were unlikely the source of *Salmonella. S. enteritidis* has been associated with eggs in a number of other countries, with the organisms found primarily inside the egg (5). Many outbreaks and cases of salmonellosis have occurred from internally contaminated eggs.

The staphylococci were not examined for enterotoxin production, as this capability was not available in the laboratory where the isolations were made and the staphylococcal culture was not retained. It was only later that the food handlers were cultured and the isolates taken to the Laboratório de Staphylococci, Fundação Ezequiel Dias, Belo Horizonte, for enterotoxin testing. The fact that most staphylococcal food poisoning outbreaks result from food contaminated by food handlers indicated that the contamination of the potato salad with *Staphylococci* was of concern. If the food handlers were colonized with enterotoxigenic staphylococci, which some were, special care should be taken to avoid contamination of the food during handling and to properly store the food once it was prepared. Such practices would reduce the frequency of staphylococcal food poisoning outbreaks currently occurring in Brazil (3, 4). One of the food handlers was colonized with staphylococci that produced SEA during the entire monitoring period and one other individual was colonized the first, second, and fourth weeks with staphylococci that produced

Table 1. Enterotoxigenicity of *S. aureus* isolated from food handlers[a]

Food handler	Week of isolation	Enterotoxin detection OSP	RPLA[b]
2	1	A	-[c]
	2	A	-
	3	-	A
	4	-	A
5	1	B	-
7	4	B	A
9	1	A	B
	2	A,B	-
	4	A,B	-

[a] - Production by membrane-over-agar with BHI medium.
[b] - Only enterotoxins negative by OSP were tested by RPLA; only positives at a 1 to 10 dilution of the culture supernatant fluid were recorded as positive.
[c] - Negative reaction for those tested.

SEA and SEB. The fact that the enterotoxin produced did not change over the four week period indicated that these staphylococci may be indigenous with the food handlers.

Salmonellosis is a very serious illness with *Salmonella* contaminated eggs frequently involved. Contamination with *Salmonella* is usually on the surface of the egg which can be removed before use of the eggs. However, in recent years *Salmonella enteritidis* has been found inside the egg in England and the United States and apparently now in Brazil. This complicates the use of raw eggs in the preparation of foods such as salad dressings, with greater care needed. The presence of 10^6 cfu of *S. aureus*/g of the potato salad demonstrates the care needed in the preparation of foods and their storage as many workers are colonized with enterotoxigenic staphylococci. The relative ease of contaminating food by food handlers is demonstrated by the many staphylococcal food poisoning incidents occurring in Brazil (3, 4).

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RESUMO

Staphylococcus aureus e _Salmonella enteritidis_ isolados de um alimento envolvido em um surto de toxinfecção alimentar

Duzentas e oitenta pessoas foram acometidas por diarréia, vômito, febre, cólica intestinal e dores de cabeça em um período de 6 a 15 horas, após a ingestão de maionese em um restaurante de Brasilia. *Salmonella enteritidis* e *Staphylococcus aureus* foram isolados a partir deste alimento. Os sintomas observados são típicos de salmoneloses como de enterotoxinas estafilococicas, com exceção da febre, que é mais comum na primeira. O período de incubação está dentro daquele observado nas salmoneloses, enquanto nas toxinfeções estafilocócicas o tempo máximo é de 6 horas. O *S. aureus* deve ter tido a sua origem dos manipuladores enquanto a *S. enteritidis*, nos ovos utilizados no preparo da maionese. Surtos de salmoneloses por *S. enteritidis*, em decorrência do consumo de ovos, foram relatados na Inglaterra e Estados Unidos. A contagem elevada de *S. aureus* no alimento é suficiente para produzir enterotoxina, porém os sintomas devem ter sido brandos, porque uma pequena quantidade do alimento deve ter sido consumida. As linhagens *S. aureus* isoladas não foram testadas quanto à produção de enterotoxina e nem a presença de enteroxina foi pesquisada no alimento. Devido à presença de *S. aureus* no alimento, os manipuladores foram submetidos a exame microbiológico e constatou-se que, dentre estes, vários eram portadores de *S. aureus* enterotoxigênicos. Estas informações são importantes, pois a maioria das toxinfecções alimentares por enterotoxina estafilocócicas é decorrente da contaminação do alimento pelos manipuladores.

Palavras-chave: Estafilococos, *Salmonella enteritidis*, toxinfecção alimentar, enterotoxina.

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OCCURRENCE OF EMERGING PATHOGENIC *VIBRIO* SPP IN SEAFOOD CONSUMED IN SÃO PAULO CITY, BRAZIL

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ABSTRACT

A number of *Vibrio* species, other than *V. cholerae* and *V. parahaemolyticus*, may cause disease in man, according to the literature. Samples of seafood consumed in São Paulo city, Brazil, were tested for the presence of these organisms, using alkaline peptone enrichment at 35°C, followed by plating onto TCBS agar. 100 samples were analyzed (56 of oysters, 20 of mussels and 24 of shrimp). Potentially pathogenic *Vibrio* spp were isolated from 89% of oysters, 50% of mussels and 17% of shrimp. Because of the high incidence of these microorganisms, we can conclude that consumers are exposed to hazardous foods, mainly if ingesting raw seafood.

Key words: *Vibrio vulnificus, Vibrio mimicus, Vibrio damsela,* seafood, *Vibrio* spp.

INTRODUCTION

Amongst *Vibrio* species, *Vibrio cholerae* group O1, the organism that causes cholera, has been the vibrio of greatest interest to public health. In the last 15-20 years, however, new *Vibrio* species have been recognized as possible causes of various types of human disease.

Vibrio cholerae non O1 have been associated with cholera-like disease and other extra intestinal infections in humans (12).

V. mimicus was the causative agent of 21 cases of diarrhoea in the USA over a 5 year period, from 1977. Fresh oysters were strongly suspected as the vehicle. In Japan, 59 cases of diarrhoea due to this organism occurred between 1984 and 1986. The isolates were from travelers' diarrhoea in 27.1% of the cases. In Japan, in an outbreak caused by *V. mimicus* affecting 13 persons, raw seafood was the vehicle of transmission (12).

V. fluvialis has been incriminated in sporadic cases as well as large scale epidemics of food poisoning in Bangladesh. In Japan, there are only two documented cases of food poisoning due to this species (12).

Shrimp and crab salad were implicated in the first reported outbreak of *V. furnissii* that occurred during a flight, in 1969. Two other additional outbreaks have occurred since then. Most of the sporadic cases were of mixed infection with *V. parahaemolyticus* (12).

V. vulnificus is unusual in its ability to produce disease by two different portals of entry: by the oral route and by wounds. Raw oysters contaminated with this bacterium have been implicated as a vehicle for gastroenteritis (7) and life-threatening septicemia in susceptible individuals (25). It is considered one of the most invasive and rapidly lethal human pathogens, with a 50% mortality rate associated with septicemic infection. Wound infections by this organism may result in amputation of limbs or death with a lower mortality rate of 25% (25).

There are few reports on emerging pathogenic *Vibrio* species in seafood in South America. The aim of this research was to study the occurrence of these vibrios with emphasis on *V. vulnificus*.

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MATERIALS AND METHODS

Materials. Samples: One-hundred raw seafood samples were examined from March 1991 to August 1993. Twenty-four shrimp (*Penaeus* spp.) samples were collected either at a shrimp industry or at fish markets showing just regular hygienic conditions. Fifty-six samples of oyster (*Crassostrea gigas*) and twenty samples of mussel (*Perna perna*) were obtained from Instituto Adolfo Lutz, a Public Health Laboratory in the same city.

Mussels and oysters were collected by public health personnel in the coastal area of São Paulo State. Sources were harvesting areas from the cities of Cananéia, Ilha Comprida, Iguape, Ilha Grande and São Sebastião.

Methods

Sample preparation **(3)**. Shellfish: oysters and mussels were washed with a scrubbing brush under flowing water. The shells were opened and for each sample, the contents of 12 shells were homogenized. 25g were removed and homogenized with 225ml of phosphate buffered saline (PBS), pH 7.2-7.5.

Crustaceans: 25g of shrimp were homogenized with 225ml of PBS, in a sterile blender jar.

Enrichment and Isolation procedure **(3)**. Ten fold dilutions (10^{-1} to 10^{-5}) were prepared in PBS. 1ml of each dilution was inoculated in three tube multiple dilution-MPN technique, using alkaline peptone water (APW). Tubes were incubated for 12-16h at 35-37°C, streaked onto thiosulphate-citrate-bile salts-sucrose agar (TCBS agar, Difco, USA) and incubated at 35-37°C for 18-24h.

Identification Both sucrose-positive and sucrose-negative colonies on TCBS agar were picked, inoculated on Kliger iron agar (Difco, USA) and on arginine glucose medium (8). The tubes were incubated at 35-37°C for 18-24h. Colonies with typical reactions on both media (alkaline slant and acid butt in both media, with the exception of *V. vulnificus* that can also show an acid slant on KIA) were submitted to preliminary biochemical tests according to Elliot *et al.* (3).

Strains were identified, afterwards, by using the API 20E system (Analytab Products, Inc., Plainview, NY.), modified for *Vibrio* identification by using 2.5% saline as an inoculum diluent (14).

RESULTS

Of the 100 samples analyzed, 64 (64%) were positive for *Vibrio* spp. These results are summarised in Table 1. *Vibrio* spp. occurred mainly in oysters (89,3%), with *V. parahaemolyticus* and *V. vulnificus* as predominant species. Shrimps showed the lowest level of contamination. Some samples harboured more than one species of *Vibrio*.

The Most Probable Number (MPN g^{-1}) of *V. vulnificus* in shellfish varied from <3 to $3.6x10^2$.

Strains of *V. mimicus* were isolated from oysters and mussels, but not from shrimps.

V. damsela was recovered from only one sample of oyster.

V. alginolyticus and *V. fluvialis* were recovered from oysters, mussels and shrimps. These species were analyzed only at the first 40 food samples.

DISCUSSION

The distribution of *Vibrio* spp. in the aquatic systems is affected by several factors, such as salinity, nutrients and temperature (10, 23). On the other hand, the occurrence of *Vibrio* in foods may be different from the findings in ecological studies.

The presence of *V. parahaemolyticus* in sea water and seafood such as crabs, shrimps, oysters, fish and lobsters have been reported by several authors (2, 13, 18, 31). In Brazil, it has been isolated from oysters (4), fishes (5), sea water (15), water-oyster ecosystem (28)

Table 1 - Occurrence of potentially pathogenic *Vibrio* species isolated from seafood in São Paulo, Brazil.

Sample type	Number of samples		Species of *Vibrio*			
	tested	positive for *Vibrio* spp. (%)*	*V. cholerae non-01*	*V. vulnificus*	*V. parahaemolyticus*	*V. mimicus*
oyster	56	50 (89.3)	2 (3.6)	29 (51.8)	49 (87.5)	7 (12.5)
shrimp	24	4 (16.7)	1 (4.2)	1 (4.2)	1 (4.2)	0 (0.0)
mussel	20	10 (50.0)	0 (0.0)	5 (25.0)	10 (50.0)	1 (5.0)
Total	100	64 (64.0)	3 (3.0)	35 (35.0)	60 (60.0)	8 (8.0)

* Some samples harboured more than one species.

and lobsters (31). In the present study, we detected *V. parahaemolyticus* in 60 out of 100 samples of seafood (60%). As already pointed out, the oysters were the most contaminated food. Rodrigues and Hofer (28) isolated *V. parahaemolyticus* in 7.94% of oysters examined, while Gelli *et al.* (4) and Leitão *et al.* (13) recovered the bacterium in 100%. In Japan, *V. parahaemolyticus* has been recognized for many years as the causative agent of over 70% of all cases of gastroenteritis. This is due to the habit of eating raw seafood (27). In the United States, outbreaks have been caused by gross mishandling of the seafood, and include improper refrigeration, insufficient cooking, cross-contamination, and recontamination.

Although Brazil is experiencing the 7th pandemia of cholera, *V. cholera* O1 was not isolated. Our results agree with Matté *et al.* (17) who also failed in recovering the organism from oysters and mussels. However, we isolated *V. cholerae* non-O1 from two samples of oysters and one of shrimp. No case of cholera had been reported by the time we isolated the strains. Matté *et al.* (17) also detected *V. cholerae* non O1 in oysters (31%) and mussels (6%).

Martins *et al.* (16) reported the isolation of *V. cholerae* O1 from sewage when there was no report on cases of cholera. Martins *et al.* (15) also detected *V. cholerae* O1 in sea water collected at Santos (SP) and surroundings. However, the detection was only possible by the immunofluorescence technique. Probably, the cells were viable but in the nonculturable state, which could also have occurred in the present research.

The recovery of *V. cholerae* non-O1 from 2 samples of oyster (3,6%) and 1 sample of shrimp (4.2%) is of concern. Strains of *V. cholerae* non O1 have shown several biologically active toxins, including CT and other toxins, cytolisins and haemolysins. These strains can present a very heterogenous mechanism of virulence, close to the one showed by *Escherichia coli* (21).

These vibrios cause food poisoning outbreaks and sporadic cases of gastroenteritis as well as septicemia and soft tissue infections (12). In the U.S. Atlantic and Gulf coastal areas, a number of infections due to non O1 serovars of *V. cholerae* have been documented (19). Almost all cases in the United States have been associated with the consumption of raw oysters.

In Japan, *V. cholerae* non-O1 was isolated from tuna sashimi during an outbreak (14). *V. cholerae* non-O1 has also been isolated in the United States (13), Spain (1), and other countries (30).

Infections due to *V. vulnificus* have occurred in Belgium, Canada, Japan and the U.S. (29). In Brazil, Zebral (33) isolated *V. vulnificus* from mussels and Rodrigues and Hofer (30) from sea water (0.51%), but not from oysters. Our results showed the presence of *V. vulnificus* in 51.8% of oysters, 4.2% of shrimp and 25% of mussels. The API 20E system proved to be very useful, with no doubt about identification.

Although the MPN per g^{-1} of food is not very high, it can be a health risk to the consumers, particularly because we have no knowledge of *V. vulnificus* infective dose.

Oysters and other seafood as well as marine and estuarine waters were shown to harbour *V. vulnificus* in various other countries (10, 25, 26, 30).

A variety of factors have been implicated as possible virulence determinants for *V. vulnificus* in animal models. These include the ability to acquire iron from transferrin, an extracellular haemolysin and a protease, and the presence of a polysaccharide capsule (27).

V. mimicus was present in oysters (12.5%) and mussels (5.0%). This is a very low incidence when compared with the results obtained by Wong *et al.* (32) in oysters (68.8%), in Taiwan. There are both toxigenic and nontoxigenic strains. The former produces a cholera-like toxin, proteases and thermostable enterotoxin. *V. mimicus* has been isolated from faeces of people with gastroenteritis due to the consumption of raw oysters in several countries - Canada, Mexico, Bangladesh (22). *V. mimicus* is widely distributed in nature and can be found in fresh as well as brackish waters.

Other vibrios isolated in the present investigation can cause disease, such as *V. fluvialis* that has been involved in cases of diarrhoea and vomiting (14).

Despite the fact that *V. damsela* was recovered from only one sample of oyster, this species is an important pathogenic bacterium because it may cause death. It has been isolated from infected wounds acquired in tropical and semitropical regions (14).

Shrimps had a lower isolation rate of *Vibrio* spp than oysters and mussels because these are filter feeders organisms and the bacterial species are part of the natural estuarine microflora. Therefore, the bivalves may accumulate all types of bacteria and viruses present in the water.

Several authors have already shown the lack of correlation between the presence of potencially

pathogenic vibrio and faecal indicators, confirming that *Vibrio* spp are autochthonous in marine and estuarine environments (1, 7, 28).

The *Vibrio* species isolated in this research are of concern because they are potentially pathogenic to man, indicating a public health problem. As Brazil has a large extension of seashore, more research is needed in order to have a good idea of the presence of the emerging pathogenic vibrios in sea water and seafood. Another factor that helps the presence of vibrios like *V. vulnificus*, for instance, is the water temperature throughout the year, particularly in the northeastern coast. To help to prevent seafood intoxications and infections, people should not eat raw or undercooked seafood. When these types of food are well cooked, bacteria are destroyed in spite of the fact that some viruses, like hepatitis A, may not be destroyed. Care should also be taken in order to prevent cross-contamination when processing foods.

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RESUMO

Ocorrência de patógenos emergentes do gênero *Vibrio* em alimentos de origem marinha consumidos na cidade de São Paulo, Brasil.

Várias espécies de *Vibrio*, outras além do *V. cholerae* e *V. parahaemolyticus*, podem causar doenças no homem. 100 amostras de alimentos de origem marinha, sendo 56 de ostras (*Crassostrea gigas*), 20 de mexilhões (*Perna perna*) e 24 de camarões (*Penaeus* spp.), foram analisadas empregando a metodologia padrão para a pesquisa desses microrganismos. Espécies potencialmente patogênicas como *V. vulnificus*, *V. mimicus* e *V. cholerae* não O1 foram isoladas de 89% de amostras de ostras, 50% de mexilhões e 17% de camarões. Devido à alta incidência desses microrganismos, pode-se concluir que os consumidores estão expostos a alimentos que representam risco à população.

Palavras-chave: *Vibrio vulnificus, Vibrio mimicus, Vibrio* spp., alimentos marinhos.

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BIOCHEMICAL CHARACTERIZATION OF A MICROBIAL GLUCOSYLTRANSFERASE THAT CONVERTS SUCROSE TO PALATINOSE

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ABSTRACT

A strain of *Klebsiella sp.* isolated from over-ripe fruits was shown to produce an intracellular glucosyltransferase that catalyses the conversion of sucrose to palatinose. The enzyme was purified by fractionation with ammonium sulfate, DEAE-Sephadex A-50 and CM-cellulose column chromatography. The purified enzyme was very active between pH 6.0 and 6.5 and the optimum temperature was 35°C. The enzyme was inhibited by Hg^{2+} and Ag^{+} yet p-chloromercuribenzoate had no effect on enzyme activity. The Km and Vmax values for the purified enzyme were 120 mM and 110 µg palatinose formed/min.ml, respectively. The molecular weight of the enzyme was 74,000 Da. The enzyme converted 4% sucrose (w/v) to isomaltulose after 64 hours incubation, with an efficiency of 86% at 25°C, pH 6.5.

Key words: *Klebsiella* sp., glucosyltransferase, palatinose production, sucrose transformation.

INTRODUCTION

Palatinose (isomaltulose, 6-α-D-glucopyranosyl-D-fructofuranose) is a reducing disaccharide with about 45% of the sweetness of sucrose but otherwise very similar in its physical and organoleptic properties. It has been reported that the sugar may be noncariogenic since much less acid and glucan are formed by *Streptococcus mutans* from palatinose than from sucrose (11). Palatinose is used in the production of speciality chocolate, chewing gum, and cookies (4).

The microorganisms *Protaminobacter rubrum* (16), *Erwinia rhapontici* (2), *Erwinia carotovora* var. *atroseptica* (9) and *Serratia plymuthica* (5,10) have been reported to transform sucrose to palatinose.

Recently we isolated a strain of *Klebsiella sp.* that produces an intracellular enzyme able to convert sucrose into palatinose by α-D-glucosyl transfer. In this research communication we report some characteristics of the purified glucosyltransferase and its production of palatinose.

MATERIALS AND METHODS

Isolation of palatinose-producing microorganism. Tissues from various over-ripe papaya fruits (approximately 1 g samples) were suspended in sterile water and streaked onto agar plates containing 1% peptone, 0.4% beef extract powder, 4% sucrose and 2% agar. All plates were incubated at 30°C for 24 - 48 h. After the appearance of growth, isolated colonies were transferred to 125 ml flasks containing 10 ml of the same culture medium devoid of agar and incubated for 48 h at 30°C, with shaking. The microbial cells were next removed by centrifugation and the cell-free culture medium examined by paper chromatography and HPLC. One strain of *Klebsiella sp.* was found to be a palatinose producer.

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Production of enzyme and purification. The selected strain of *Klebsiella* sp. was inoculated into 500 ml Erlenmeyer flasks containing 100 ml culture medium as described above and incubated for two days at 25°C with shaking (200 rpm). Next, 500 ml of culture medium were centrifuged; the cell pellet obtained was washed three times with deionized water by centrifugation. Finally, the cells were suspended in 10 ml of 0.1M citrate-phosphate buffer, pH 6.0, and disrupted by sonication (Biosonik IV Bronwill Scientific) on an ice bath (45 seconds; 40khz). The sonicated preparation was centrifuged for 10 min at 10,000 rpm; the supernatant containing the enzyme was diluted to 50 ml and solid ammonium sulfate added to this solution to give 80% saturation. The precipitate was collected by centrifugation and dissolved in 5 ml of distilled water. The enzyme solution was dialyzed against distilled water for 48 h and then against 0.01M citrate phosphate buffer, pH 6.0. The dialyzed material was applied to a column (2.5 cm x 30 cm) of DEAE-Sephadex equilibrated with 0.05M citrate-phosphate buffer, pH 6.0, and eluted with a further 70 ml of the same buffer. A step-wise elution was then carried out with 100 ml volumes of 0.1M, 0.2M, 0.3M, 0.4M and 0.5M NaCl in 0.05M citrate-phosphate buffer, pH 6.0. The flow rate was about 11 ml per hour. Eluates were collected in 5.5 ml fractions.

The elution pattern is shown in Fig. 2. The enzymatically active fractions were pooled, dialyzed against water and concentrated by ultrafiltration with a collodion bag (Sartorius 13,200). The enzyme solution was applied to a column (2.5 cm x 35 cm) of CM-cellulose equilibrated with 0.05M citrate-phosphate buffer, pH 6.0, and the column was washed with 100 ml of the same buffer. The column was eluted successively with the following buffer system:

a) 100 ml equilibrating buffer containing 0.1M NaCl;
b) 100 ml equilibrating buffer containing 0.2M NaCl.

The fractions exhibiting glucosyltransferase activity were pooled (Fig. 3) and the enzyme solution dialyzed against distilled water and lyophilized.

Quantitative determination of proteins. Protein concentrations for the various enzyme purification steps were measured by the method of Lowry *et al.* (8) using bovine serum albumin as standard. The protein concentration of each fraction during column chromatography was estimated by absorbance at 280 nm.

Assay of glucosyltransferase activity. A reducing palatinose was the only product of sucrose produced by the isolated *Klebsiella* sp. Enzyme activity was therefore determined by incubating a mixture of 900 µl of 4% sucrose in citrate-phosphate buffer 0.1M, pH 6.5, with 100 µl of enzyme solution (30°C; 20 min). The reducing sugars formed were determined by the method of Somogyi (13). One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 µmole of palatinose per min under the assay conditions.

Paper chromatography. Descending paper chromatography was run on Whatman filter paper n° 1 (0.25M boric acid treated) for 16 hr with a solvent system of ethyl acetate-isopropanol-water (6:3:1 by vol). Sucrose and palatinose were used as standards. Sugar spots were detected with aniline-diphenylamine-phosphoric acid (12).

HPLC. High performance liquid chromatography was performed with a Waters model M6000 A pump, Waters RI detector model R401 and ZORBA-NH_2column (4.6x250 mm). The samples were eluted with acetonitrile:water (75:25 v/v) at a flow rate of 1 ml/min. Sucrose and palatinose were used as standards.

Determination of molecular weight. *1) Gel filtration.* The molecular weight of the enzyme was estimated by Sephadex G-200 (2 x 100 cm) gel filtration according to the method of Andrews (1). The column was equilibrated with 0.05M Tris-HCl buffer pH 7.5 containing 0.1M KCl.

Purified glucosyltransferase and protein standards (bovine serum albumin MW. 67,000; egg albumin MW. 43,000 and lysozyme MW. 14,000) were eluted with the same buffer at a flow rate of 16 ml h^{-1} and 4 ml fractions were collected. Void volume (Vo) was measured with blue dextran 2000.

2) SDS - Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-PAGE was performed by the method of Weber and Osborn (15) using an electrophoresis calibration kit for molecular weight determination (Pharmacia). Protein bands were stained with Coomassie brilliant blue.

Effect of Substrate on Activity. The concentration of substrate during enzyme estimation by the standard procedure was varied and the results

treated according to the method of Lineweaver and Burk in order to calculate the Km value.

Effect of pH on glucosyltransferase activity. Reaction mixtures containing 100 µl of purified enzyme and 900 µl of 4% sucrose solution were incubated for 20 min at 35°C at pH values varying from 2.6 to 8.0.

Effect of temperature on glucosyltransferase activity. Reaction mixtures containing 100 µl of purified enzyme and 900 µl of 4% sucrose solution in citrate-phosphate 0.05M pH 6.0 were incubated for 20 min at temperatures ranging from 25°C to 60°C.

Effect of temperature on the conversion of sucrose to palatinose. The effect of temperature on the conversion of sucrose to palatinose was investigated by incubating 50 ml of 4% sucrose in 0.05M citrate-phosphate buffer, pH 6.5, with 1.2 units of the purified enzyme at 25°C, 30°C and 35°C.

RESULTS AND DISCUSSION

Paper chromatography of the cell suspension plus sucrose reacting mixture after 1 hr incubation at 30°C yielded two spots on application of the aniline-diphenylamine-phosphoric acid spray reagent. The first green-yellow spot was produced by a 1,6-linked glucosaccharide and corresponded to palatinose.The other purple brown spot corresponded to sucrose. The presence of palatinose and sucrose in the reaction mixture was confirmed by HPLC as shown in Fig. 1. This experiment demonstrates that the glucosyltransferase was located in the microbial cells.

Purification of the enzyme. The enzyme purification process is summarized in Table 1. An approximate 12.1 fold purification was attained with a 6.5% yield recovery from the crude extract. The enzyme was adsorbed on DEAE-Sephadex A-50 at pH 6.0 (Fig. 2). CM-cellulose column chromatography was used for the next purification step; as shown in Fig. 3, the glucosyltransferase was not retained by the column and appeared in the early fractions associated with the void volume.

Enzyme characterization. The molecular weight of the glucosyltransferase estimated by Sephadex G-200 gel filtration was approximately 74,000 Da (Fig. 4). The enzyme gave a single protein band by SDS-gel electrophoresis, with an estimated molecular weight of 74,000 Da.

A purified glucosyltransferase from *Serratia plymuthica* ATCC 15928 was shown to have a molecular weight of 79,500 by gel filtration (10).

The Km and Vmax values of the purified *Klebsiella sp* enzyme for sucrose were 120 mM and 110 µg palatinose formed/min./ml, respectively (Fig. 5). This Km value was greater than that described by McAllister *et al.* for a purified enzyme with glucosyl transfer activity isolated from *Serratia phymutica* (65.3 mM) (10). Immobilized cells of *Erwinia rhapontici* (2) and *Protaminobacter rubrum* (14) were also shown to have glucosyltransferase activity with Kms of 350 mM and 140 mM, respectively.

The optimum pH of the purified enzyme was between pH 6.0 and 6.5 (Fig 6) and the optimum temperature was about 35°C for an incubation time of 20 min (Fig. 7).

The glucosyltransferase of *Serratia phymuthica* showed optimum activity at 30°C and pH 6.0 (10).,

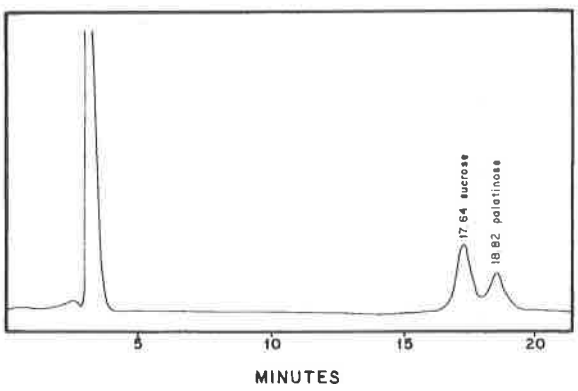

Figure 1. HPLC for Conversion of Sucrose to Palatinose.

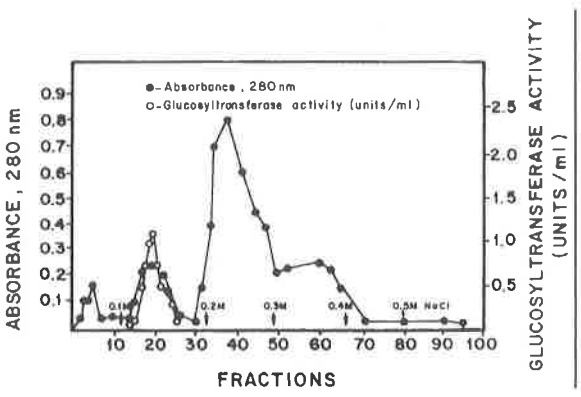

Figure 2. Purification of glucosyltransferase by DEAE -

Table 1. Purification of a glucosyltransferase from *Klebsiella* sp.

Purification step	Vol. (ml)	Glucosyltransferase activity (units/ml)	Total units	Protein (mg/ml)	Sp. act (units/mg)	Yield (%)	Purification
Cell free extract	50	2.95	147.5	205.5	0.014	100.0	1.0
Ammonium sulfate fractionation	15	7.00	105.0	470.0	0.015	71.2	1.1
DEAE-Sephadex A-50 column chromatography	55	0.21	11.5	2.3	0.091	7.8	6.5
CM-cellulose column chromatography	16.5	0.58	9.5	3.4	0.170	6.5	12.1

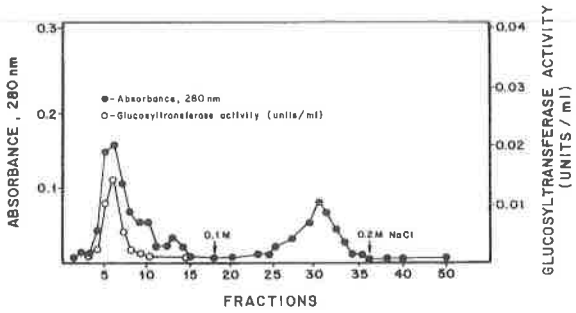

Figure 3. Purification of glucosytransferase by CM-cellulose

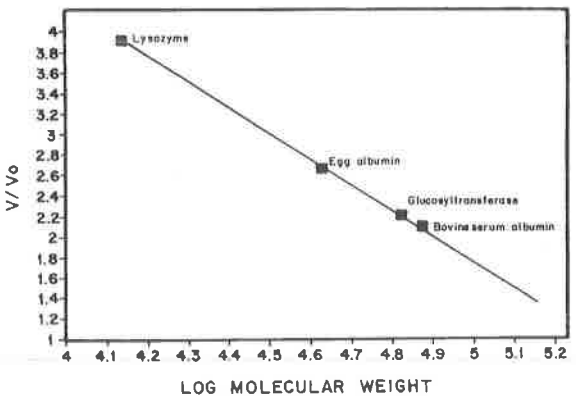

Figure 4. Estimation of the Molecular Weight of the purified glucosytransferase by gel Filtration on Sephadex G-200.

whereas the optimum activity for isomaltulose synthesis at 30°C using immobilized cells of *Erwinia rhapontici* (2) and *Protaminobacter rubrum* (14) was detected at pH 7.0 and pH 5.5, respectively.

The effect of metal ions on enzyme activity was investigated. It was found that 1 mM of either Hg^{2+} or Ag^{+} in the reaction mixture totally inactivated the enzyme, whereas no inhibition was observed in the

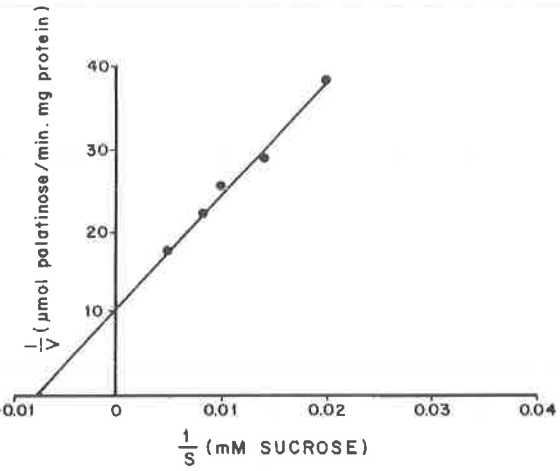

Figure 5. Lineweaver - Burk plot of purified *Klebsiella* sp glucosyitransferase activity on sucrose.

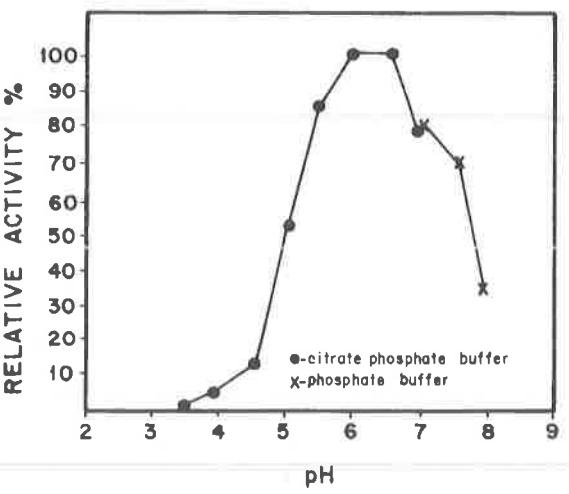

Figure 6. Effect of pH on Glucosyltransferase.

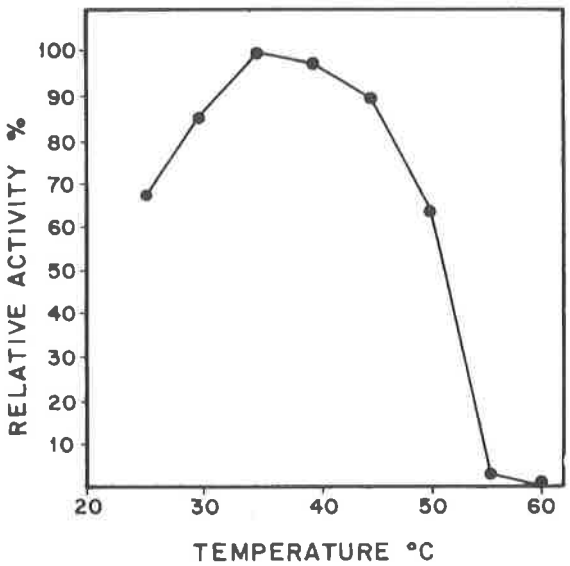

Figure 7. Effect of Temperature on Enzyme Activity.

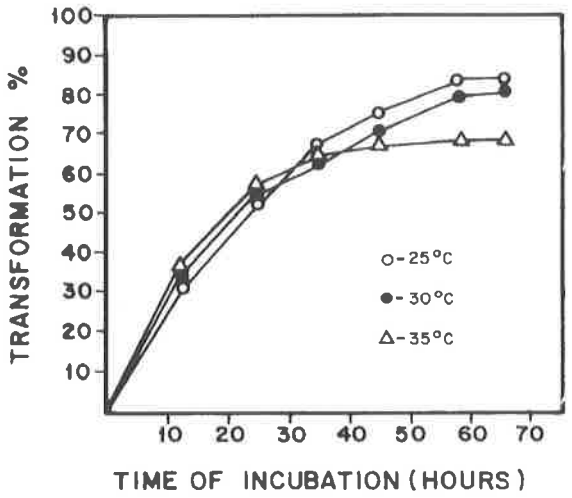

Figure 8. Time Course of Palatinase Formation at Various Temperatures.

presence of $CuSO_4$, KCl, $MgSO_4$, $Pb(CH_3COO)_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, $BaCl_2$ or $FeCl_3$.

Hayashi *et al.* (6) reported that a glucosyltransferase from *Aureobasidium* sp. ATCC 20524 which produces panose and isomaltulose from sucrose was strongly inhibited by 1mM Hg^{2+}, Ag^{+}, Al^{3+}, Ni^{2+} or Cu^{2+}.

Since no inhibition of the purified enzyme was observed with EDTA, it seems that metals are not essential for the *Klebsiella* sp. glucosyltransferase to convert sucrose to palatinose.

Hayashi *et al.* (6) also reported that EDTA did not inhibit enzyme activity of a glucosyltransferase from *Aureobasidium* sp. ATCC 20524. Hirayama *et al.* (7) reported that metals are not essential for the transfer action of a fructosyltransferase from *Aspergillus niger* ATCC 20611, as no inhibition of this purified enzyme was observed in the presence of EDTA.

The glucosyltransferase activity of *Klebsiella* sp. was not inhibited by SH group reagents such as p-chloromercuribenzoate. Hayashi *et al.* (6) verified that the glucosyltransferase of *Aureobasidium* sp. ATCC 20524 was slightly inhibited by p-chloromercuribenzoate.

Fructosyltransferase activity of *Aspergillus niger* ATCC 20611 was not inhibited by p-chloromercuribenzoate (7).

Effect of temperature on the conversion of sucrose to palatinose. The conversion of sucrose to palatinose at 25°C, 30°C and 35°C is shown in Fig. 8. A maximum conversion of 86% was reached when the enzyme was assayed at 25°C. The glucosyltransferase of *Serratia phymuthica* converted 40% (v/v) of sucrose to isomaltulose with an efficiency of 87% (10).

RESUMO

Caracterização bioquímica de glicosiltransferase microbiana que converte sacarose para palatinose

Foi isolada de fruta deteriorada uma linhagem de *Klebsiella sp* que transforma sacarose em palatinose. Foi verificado que a linhagem produz uma glicosiltransferase intracelular que transforma sacarose para palatinose. A enzima de *Klebsiella sp* foi purificada por fracionamento com sulfato de amônio e cromatografia em colunas de DEAE-Sephadex A-50 e CM-celulose. A enzima purificada apresentou atividade ótima a 35°C e na faixa de pH 6,0 a 6,5. A enzima foi inibida por Hg^{2+} e Ag^{+}, mas não foi inibida por p-cloromercuribenzoato. Os valores de Km e Vmax da enzima purificada foram respectivamente 120 mM e 110 μg palatinose formada por minuto por ml de enzima para o substrato sacarose. O peso molecular da enzima foi estimado em 74.000 Da. A enzima converteu 4% (p/w) de sacarose para isomaltulose com uma eficiência de 86% a 25°C, pH 6,5.

Palavras-chave: *Klebsiella* sp, glicosiltransferase, produção de palatinose, transformação de sacarose.

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PRODUCTION OF GLUCOAMYLASE BY FED- BATCH CULTURE OF *ASPERGILLUS AWAMORI* NRRL3112*

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ABSTRACT

Glucoamylase production by *Aspergillus awamori* NRRL3112 was 92% higher in fed-batch culture relative to the traditional batch fermentation for runs with 20g/l of total carbon source from cassava flour. An analysis of the specific production rate shows that this was due to a reduction in the repressive effect of glucose, as the inductive effect of the polysaccharide concentration was also reduced.

Key words: induction, repression, enzyme synthesis, process.

INTRODUCTION

Glucoamylase synthesis by *Aspergillus sp.* is regulated by catabolic induction and repression. Although this enzyme is produced in the presence of glucose and fructose as carbon source, polysaccharides lead to higher yields. The production of this enzyme by *Aspergillus awamori* NRRL3112 in culture medium containing cassava starch or maltose was shown to be 60% higher than in medium containing glucose (2, 3, 9).

The source of nitrogen also influences enzyme synthesis. When using peptone instead of NH4Cl, microbial growth was strongly enhanced and higher production levels of glucoamylase were obtained, although a reduction in specific production relative to cells was also reported (12).

Many other factors, like pH, specific respiration rate, type of culture, type of microorganism and its method of preservation are also important to enzyme synthesis (5, 6, 8).

Since polysaccharides are converted to glucose during culture, production strategies must be introduced in order to maintain low glucose concentrations and a certain amount of polysaccharides, which ensure a low repressive effect and a mild inductive effect, respectively (4).

In batch cultures with cassava starch, glucoamylase production is strongly induced at the start of cultivation but becomes repressed after complete hydrolysis of the polysaccharide to glucose. It was possible to increase productivity by means of a pulse fed batch process, though the final glucoamylase activity remained the same as with the conventional batch fermentation (6).

This work shows some results on glucoamylase synthesis by *Aspergillus awamori* NRRL3112 using a fed-batch approach that was compared to the traditional batch process. The latter presents higher glucose concentration levels and therefore a greater inhibitory effect on enzyme synthesis.

MATERIALS AND METHODS

Microorganism and inoculum

Spores of *Aspergillus awamori* NRRL3112 stored in tubes with sterile soil were used throughout this study (7). The strain was cultured in an Erlenmeyer flask (1L capacity) containing 200 ml of the culture medium described below. Incubation was carried out for 24 h at 35°C in a rotative shaker (250 min^{-1}). The

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culture obtained had 1.8g dry cell/l and was used as inoculum for the one batch and two fed-batch runs analyzed in the present study.

Culture medium. The medium of the inoculum and batch run contained an initial total reducing sugars concentration (TRSo) of 20 g/l. The TRS concentration was adjusted adding an adequate volume of cassava flour syrup (1). For fed-batch runs, the TRSo was 2g/l; 18 g/l were then transferred through the feed medium to give a total concentration (TRSt) of 20g/l. Since the TRS concentration of the feed medium was high (370 g/l), the total volume fed to the system was small (only about 5% of the culture broth volume) and this allowed all the results to be expressed as concentrations. The culture medium also contained the following nutrients (g/l): yeast extract 0.1; $(NH_4)_2SO_4$ 5.0; $Na_2HPO_4.12H_2O$ 3.78; KH_2PO_4 3.5; $MgSO_4.7H_2O$ 0.5.

Culture conditions. The batch and fed-batch runs were performed in a 15-l bioreactor LSL Biolafitte SA France. The operation conditions were as follows: reaction volume=10 L; inoculum fraction (volume of the shaker culture / volume of culture medium in the reactor) =0.1; agitation rate=700 m^{-1}; air supply =10 L/min; head pressure=0.2atm; pH=4.0; temperature=35°C.

Runs. One batch run (run 1) and two fed batch runs (runs 2 and 3) with feeding rates (f_s;gTRS/h) of 16.4 and 22.5, respectively, were performed.

Analytical techniques. Samples collected periodically from the fermenter were evaluated for: dry cell mass (X); glucose concentration (G) by the glucose-oxidase method; total reducing sugars (TRS), determined as glucose after enzymatic hydrolysis of the polysaccharide in the sample (10); glucoamylase activity (A), (11). One glucoamylase activity (U) was defined as the quantity of enzyme that releases 1 g of glucose per hour in the presence of 4% (w/v) soluble starch solution, at 60°C and pH 4.2. The concentration of polysaccharide (PS) corresponds to TRS minus G.

RESULTS AND DISCUSSION

The characteristics of the batch and fed-batch runs are specified in Table 1 and the results are summarized in Table 2. Figure 1 shows the typical time course of the variables measured and the specific production rate of glucoamylase (μ) for the batch culture (run 1). The high μ_a values at the beginning of culture result from the high PS concentration present. However, after 10 h, the μ_a value strongly decreased because by

Table 1. Runs identification

BATCH RUN: Run 1 (TRS_0 = 20g/L) FEED BATCH RUNS (TRS_1 = 20g/L)			
Run	fs	Θo	$\Delta\Theta$
	(gTRS/h)	(h)	(h)
2	16.4	4	11
3	22.5	0	8

Table 2. Characteristics of the fed-batch runs.

Run	Amax (U/L)	P_A (U/Lh)	μ_{amax} (U/gh)	t_1 (h)	t_2 (h)	$\mu'a/\mu amax$
1	500	29	24	2	14	0.33
2	840	40	13	8	12	1.00
3	960	48	21	12	12	1.00

NOMENCLATURE

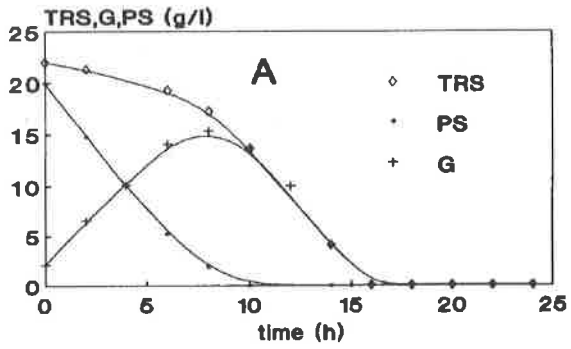
- Amax: maximum glucosmylase activity
- P_A: glucoamylase producitivy
- P_A: $\frac{Amax - A_0}{t'f}$ A_0: glucoamylase activity at t=0; t'f culture time at Amax
- μ_{amax}: maximum specific production rete of the glucoamylase
- μ_a: specific production rate of glucoamylase

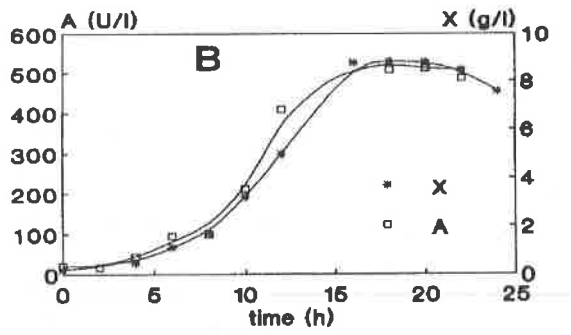
$$\mu a = \frac{1}{X}\frac{dA}{dt}$$

- t_1: culture time at μ_{amax}
- t_2: culture time at $\frac{X}{Xmax} = 0.8$

Xmax: maximum cellular concentration

- $\mu'a$: specific production rate of the glucoamylase at $t = t_2$





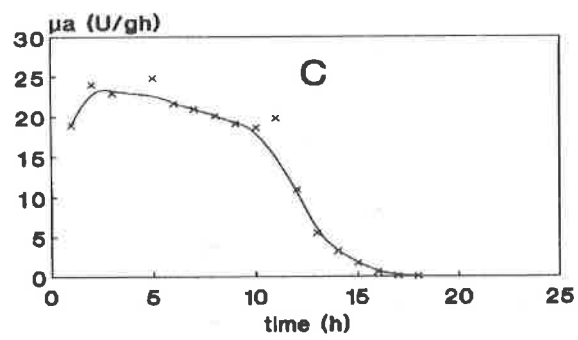
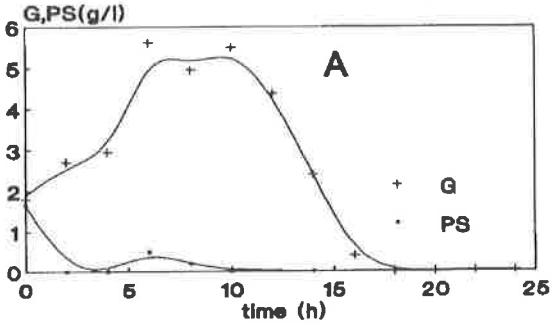
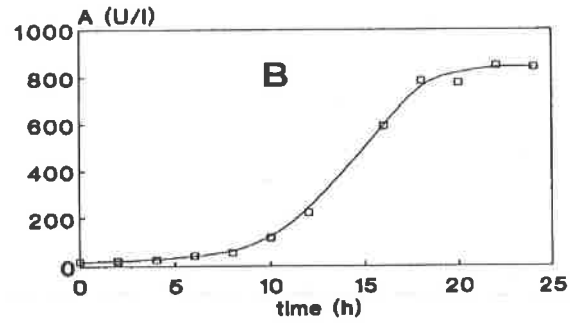


Figure 1. Time course profiles of total reducing sugars, glucose and polysaccharide concentrations (a); cellular concentration and glucoamylase activity (b); and specific production rate (c) for Run 1.





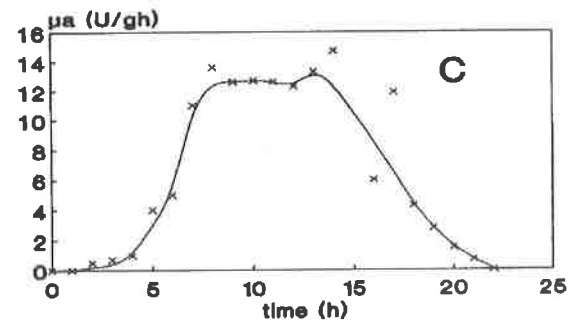
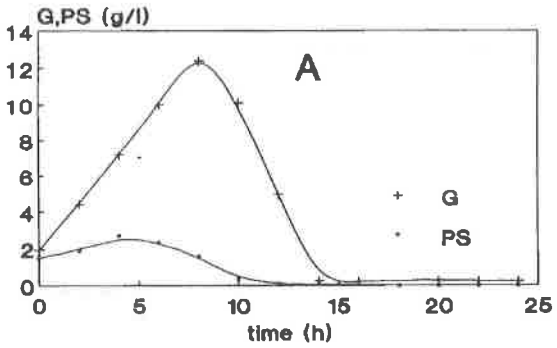
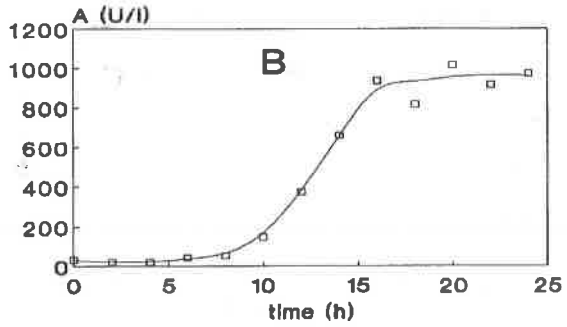


Figure 2. Time course profiles of glucose and polysaccharide concentrations (a); glucoamylase activity (b); and specific production rate (c) for Run 2.





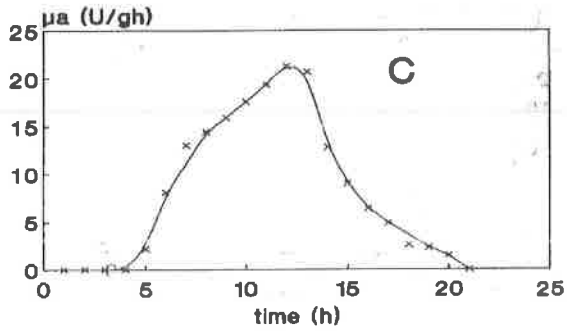


Figure 3. Time course profile of glucose and polysaccharide concentrations (a); glucoamylase activity (b); and production rate (c) for Run 3.

then the carbon source was almost exclusively glucose.

The low feeding rate in run 2 led to glucose concentrations lower than those obtained for the batch run. As can be observed in Figure 2, the maximum glucose concentration in run 2 was only 5 g/l whereas in run 1 it reached 15 g/l . On the other hand, positive PS levels were observed for run 1 until 8 h of culture, yet almost no PS was detected in the fed-batch culture due to immediate hydrolysis of added polysaccharide by glucoamylase. The PS profiles are mirrored in the μ_a values, which reached a maximum of 24 U/gh and of only 13 /gh in the batch and fed-batch runs, respectively. However, as shown in Table 2, this maximum μ_a for the batch run occurred at the start of fermentation when X was low, decreasing thereafter to a μ'_a/μ_{amax} value of 0.33 when X was high (80% of Xmax). The fed-batch maximum μ_a was constant during 14 h of culture, with X corresponding to 80% of maximum X. This value is higher than the one recorded for the batch run during the same time period. Maximum activity (Amax) at fed-batch culture (840 U/l) was 68% higher and productivity was 60% higher than the corresponding values for the batch culture. This increased production using the fed-batch approach is related to a reduction in glucose levels, since the concentration of polysaccharide was lower than that in the batch culture.

Growth was not influenced by glucose profiles. For all the runs, maximum cellular concentration and cell productivity were about 8.5 g/l and 0.5 g/l.h, respectively.

Run 3 differed from run 2 in its higher f_s value and the fact that substrate feeding was initiated at the start of culture, as described in Table 1. These two distinct features of run 3 led to higher glucose values relative to run 2 but still lower than those reached during batch fermentation.The maximum glucose concentration for run 3 was 12 g/l and its level of PS was higher than that for run 2. The combination of both higher glucose and PS concentrations in run 3 resulted in a net positive gain in microbial production capacity, with an Amax value of 960 U/l corresponding to a 92% increase relative to the batch process. The μ_a profile (Figure 3) illustrates these points.

Although the greatest production of enzyme was attained with run 3, the low glucose concentration observed in run 2 is an interesting finding concerning cultures with higher TRSt values, since high levels of X and A indicate fast polysaccharide hydrolysis and

thus glucose as the sole source of carbon in the medium.

The data obtained show that glucoamylase production can be enhanced in fed-batch fermentation relative to the traditional batch fermentation due to a reduction in the inhibitory effect of glucose.

RESUMO

O processo descontínuo alimentado na produção de amiloglicosidase por *Aspergillus awamori* NRRL3112

A produção de amiloglicosidase por *Aspergillus awamori* NRRL3112 em processo descontínuo alimentado é 92% superior em relação ao processo descontínuo em cultivos com 20 g/l de concentração total de fonte de carbono oriunda de farinha de mandioca. Uma análise da velocidade específica de produção mostra ser este resultado decorrente da redução do efeito repressivo causado pela glicose, já que o efeito indutivo, relacionado à concentração de polissacarídeo, também foi reduzido.

Palavras-chave: indução, repressão, síntese de enzimas, processo.

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