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Endereço

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05508 – São Paulo – SP – Brasil
Fone: (011) 813-9647 – Telex: 011 35085 –

REVISTA DE MICROBIOLOGIA
Depto. de Microbiologia – ICB II – USP
Av. Prof. Lineu Prestes, 1374 – Cid. Universitária
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INDUCTION OF HUMAN AMNIOTIC INTERFERON BY STRAINS OF PARAMYXOVIRUS: ROLE OF DEFECTIVE INFERFERING PARTICLES

Regina Maria Bringel Martins[1]
Romain Rolland Golgher[2]

SUMMARY

The Cantell strain of parainfluenza virus 1 needs defective interfering (DI) particles for the production of human interferon (IFN). The Mill Hill strain of Newcastle disease virus apparently does not require them and it is a more potent IFN inducer. To investigate this contrasting behaviour, serial egg passages were done in low and high multiplicity of infection (moi) with these viruses. High multiplicity of infection favor the generation of DI particles. After each passage, human amniotic membrane IFN (IFN-AM) was induced and viral hemagglutinating and infectivity titers were determined.

The Cantell strain showed a 10,000 reduction in infectivity titer with the high moi passages titers of IFN-AM produced with the low moi passages were about 300 units/ml, but the inducing capacity rose from 1,200 U/ml to 4,200 U/ml with the high moi passages. No difference was seen with the Mill Hill strain in the infectivity titer and in the yields of IFN-AM (about 12,000 U/ml), with the serial passages in low or high multiplicities. Standard and DI Cantell particles are necessary to optimize IFN-AM induction. With the Mill Hill strain, no DI particle formation could be demonstrated and no modification on the titers of IFN-AM could be observed with the addition of Cantell DI particles.

Key words: Human amniotic/Interferon/DI Particles/Paramyxovirus.

INTRODUCTION

Defective interfering (DI) particles represent a class of virus deletion mutants that require nondefective, co-infecting homologous standard virus particles to multiply. In addition, DI particles usually interfere with the growth of the standard particles. They arise spontaneously and are amplified to detectable levels by repeated passage of virus stocks at high multiplicities of infection. Under these conditions, a virus preparation shows a decreased infectivity in relation to the total number of virus particles or hemagglutinin titer. Sendai and Newcastle disease virus form DI particles when they are grown in embryonated eggs (Re, 1991).

The role of DI particles differs on the induction of interferon (IFN) according to the virus used. With vesicular stomatitis and Sindbis viruses, these particles induce IFN by themselves (Marcus & Sekellik, 1977; Fuller & Marcus, 1980). Sendai virus DI particles, however, are unable to do it (Portner & Kingsbury, 1971).

Nevertheless, the Cantell strain of Sendai virus needs DI particles mixed in a certain propor-

Laboratório de Vírus, Departamento de Microbiologia, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil.

1. Departamento de Microbiologia, IPTESP, Universidade Federal de Goiás, Goiânia, GO, Brasil.
2. Present address - Centro de Pesquisas René Rachou, FIOCRUZ, Caixa Postal 1743, 30190, Belo Horizonte, MG, Brasil.

tion with standard particles for the production of maximal titers of human IFN by Namalwa cells, a lymphoblastoid cell line. This virus strain is widely employed for the mass production of natural human IFN (Cantell et al., 1981; Mizrahi, 1983; Matsumoto et al., 1986). The Mill Hill strain induces substantially higher amounts of human IFN in cultures of leukocytes and of amniotic membranes than the Cantell strain and apparently does not form DI particles (Martins et al., 1989). Recently, the Mill Hill strain was found to be a Newcastle disease virus strain (R. M. B. Martins, M. Siqueira, J. P. Nascimento, unpublished data).

Therefore, the role of the DI particles in the induction of human IFN was still an open question and we have investigated further the ability of the Mill Hill strain to generate DI particles and to induce human amniotic IFN (IFN-AM), in comparison with the Cantell strain. For this, serial egg passages were done with the two strains, using a high multiplicity of infection (moi), which usually causes the appearance of DI particles, and low moi passages, when standard particles are preferably generated. Then, the capacity of IFN-AM induction, the infectivity and the hemagglutinating titers of the serial passages were determined as well as the result of the addition of Cantell DI particles on the yields of IFN-AM.

Results described in this paper show that no DI particle formation could be demonstrated with the Mill Hill strain, although this was easily done with the Cantell strain. In addition, DI particles are essential for the production of optimal titers of IFN-AM with the Cantell strain, whereas they are not necessary for the excellent levels induced by the Mill Hill strain.

MATERIAL AND METHODS

Cells - The African green monkey kidney cell line Vero (supplied by the American Type Culture Collection) was grown in MEM containing 5% (v/v) fetal calf serum and antibiotics.

Viruses - The Cantell (obtained from Dr. K. Cantell, Helsinki, Finland) and Mill Hill (supplied by Dr. R. D. Machado, Rio de Janeiro, Brazil) strains were grown in embryonated hen's eggs. Eight passages were done with undiluted alantoic fluid (high moi) to obtain DI particles (Johnston, 1981) and alantoic fluid diluted one to 10 million (low moi) to favor the growth of standard particles. DI particles were purified from alantoic fluids of the eighth passage in sucrose gradients (Johnston, 1981) and quantified by hemagglutination. The purified preparation had 10,240 hemagglutination (HA) units/ml. The fifty per cent egg infectious dose (EID50) and HA unit titrations were performed by standard techniques (Lennette & Schmidt, 1979). Encephalomyocarditis virus (EMC) was donated by Dr. I. Kerr, London, England and the stock was grown in L929 cells.

Induction of IFN - Human amniotic IFN was produced essentially as described (Fournier et al., 1967): the amnion was separated from the placenta, washed and 5 g of the tissue were infected with 2.0ml of the Cantell or Mill Hill strains and incubated for one hour of adsorption. Then 40 ml of MEM medium containing 0.1% of human plasma (previously adsorbed with polyethylene glycol) and antibiotics were added and the flasks were reincubated. After 24 h and 48 h, the media were collected, adjusted to pH 2 and their IFN yields were assessed.

The role of DI particles was examined by induction with the eighth passage at low multiplicity of infection of the Cantell or Mill Hill strains using 0.4 ml/g of alantoic fluid per gram of tissue. At the same time, 0.1, 0.25 and 0.5 ml of a purified preparation of DI particles of the Cantell strain was added. This preparation had a titer of 10,240 HA per ml.

Interferon assay - The IFN dilutions were incubated with Vero cells in microtiter plates for 24 h before addition of the EMC virus. The 50% cell protection endpoints were determined upon 48 h of infection (Ferreira et al., 1979). All interferon titers are given in laboratory units (interassay variation coefficient - 36%). The human alpha IFN standard MRC 69/19 (5,000 units/ml) had and average titer of 25,000 laboratory units per ml.

RESULTS

The serial egg passages of the Cantell strain with the high moi resulted in a reduction of its infectivity titer from $10^{7.4}$ to $10^{4.3}$ /ml, while the HA titer dropped four times. In the same moi, the Mill Hill strain increased its infectivity 10 times with no significant change in the HA concentrations. However, with the low moi passages, the infectivities of the two strains rose about 10 times but the HA enlarged accordingly only with Cantell strain (Table 1).

Low IFN-AM titers were obtained when the infection of amnions was done with the Cantell strain in all passage levels in a low moi. However, the same strain, in a high moi, showed a signifi-

cant increase in the IFN induced with the egg passage level. On the other hand, no significant difference could be observed in the production of the IFN-AM with the Mill Hill strain using the high or low moi passage levels. The quantities of IFN-AM induced by the Mill Hill strain were much higher to those obtained by the infection with the Cantell strain with any moi (Table 1).

TABLE 1 - Cantell and Mill Hill strains serially passaged in eggs: infectivity, hemagglutination and induction of human amniotic interferon.

Passage number	EID_{50}[b]	Multiplicity of infection[a]				
		High HA[c]	IFN[d]	EID_{50}	LOW HA	IFN
		Cantell Strain				
1	$10^{7.4}$	20,480	1,200	$10^{7.6}$	2,560	700
2	$10^{6.9}$	20,480	1,500	$10^{7.8}$	5,120	300
3	$10^{6.8}$	20,480	1,800	$10^{8.4}$	10,240	250
4	ND	20,480	1,800	ND	5,120	300
5	$10^{6.1}$	20,480	2,400	$10^{8.6}$	10,240	250
6	$10^{4.5}$	20,480	4,000	ND	20,480	200
7	ND	5,120	4,000	ND	20,480	300
8	$10^{4.3}$	5,120	4,200	$10^{8.6}$	20,480	350
		Mill Hill Strain				
1	$10^{8.6}$	320	12,000	$10^{8.8}$	640	10,500
2	$10^{8.6}$	320	14,000	$10^{8.9}$	640	14,000
3	$10^{8.8}$	320	9,800	$10^{8.8}$	640	14,000
4	ND	640	14,800	ND	640	16,000
5	$10^{8.8}$	640	11,500	$10^{8.9}$	640	14,000
6	$10^{8.9}$	640	10,200	$10^{9.1}$	640	11,000
7	$10^{9.3}$	640	12,400	$10^{9.2}$	640	12,600
8	$10^{9.6}$	320	16,000	$10^{9.6}$	640	14,800

a - High - passages done with undiluted alantoic fluid. Low - passages done with alantoic fluid diluted 10^{-7}.
b - Egg infectious dose 50%ml.
c - Hemagglutinating units/ml.
d - Interferon produced by 5g of amion. Numbers represent titers of 24 and 48h added (u/ml).

Since no evidence of DI particle formation was seen with the Mill Hill strain, the role of these particles was investigated with DI particles derived from the Cantell strain. A purified preparation was used to determine its IFN-inducing ability. Amniotic cultures were inoculated with the eighth egg passage of the Cantell or Mill Hill strain, both diluted 10^{-7} and mixed with several concentrations of DI particles. The presence of these particles increased the IFN titer induced with the Cantell but not with the Mill Hill strain (Table 2). The quantity of IFN

TABLE 2 - Effect of the addition of Cantell DI particles on the yields of human amniotic interferon.

Strain	DI particles HA (units/ml)	IFN Titer (u/ml) 24h	48h
Cantell	0	500	1,600
	1,024	6,400	2,400
	2,560	9,600	6,400
	5,120	9,600	2,400
Mill Hill	0	11,200	6,400
	1,024	9,600	3,600
	2,560	9,600	2,600
	5,120	9,600	4,600

IFN induction was done by the 8th passage at low multiplicity of infection of Cantell or Mill Hill strain. Alantoic fluid (0.4ml) was added per gram of tissue. Different volumes of a preparation of purified Cantell DI particles (10,240 HA units/ml) were used.

rose rapidly to a maximum that corresponded to 2,560 HA units but then declined with further addition of DI particles (10,240 and 20,480 HA units). The preparation of DI particles alone was unable to induce IFN-AM (results not shown).

DISCUSSION

The infectivity titer of the Cantell strain when passaged at a high moi in eggs diminished considerably (Table 1) reaching a 10,000 reduction on the sixth passage, with no major drop on the hemagglutinating titer. It is known that passages with undiluted virus favors the amplification of DI particles with reduced infectivity (Kingsbury & Portner, 1970; Johnston, 1981). With the Mill Hill strain, however, this did not happen and there was no clear evidence for the formation of DI particles. This strain, passaged either with low or high moi, induced much higher IFN-AM titers than the Cantell strain (Table 1). The comparative higher potency of the Mill Hill strain was reported (Martins et al., 1989).

DI particles mixed with a certain proportion of standard particles (low moi passages) of the Cantell strain are needed for the induction of optimal titers of IFN-AM (Table 2) and Namalwa IFN (Johnston, 1981). With IFN-AM, maximal levels were produced with an input multiplicity of 100 DI particles per cell (2,560 HA units) on the assumption that one HA unit equals one million particles (Kingsbury & Portner, 1970) and 1 g of amnion has 5 million cells (J. R. Santos, personal communication). Purified DI particles by themselves were not able to induce IFN-AM (data not shown), chick IFN (Portner

& Kingsbury, 1971) or Namalwa IFN (Johnston, 1981). No effect of DI particles was seen with the Mill Hill strain (Table 2).

The results showed that for the same tissue, DI particles are important (Cantell strain) or not (Mill Hill strain) for IFN induction, indicating the complex nature of this phenomenon. Investigations on its nature will contribute to the knowledge of the viral induction of IFNs.

RESUMO

Indução de interferon humano de membrana amniótica: papel de partículas defectivas interferentes.

A amostra Cantell de vírus parainfluenza 1 necessita de partículas defectivas interferentes (DI) para a produção de interferon (IFN) humano. A amostra Mill Hill do vírus da doença de Newcastle aparentemente não as requer, embora seja um indutor de IFN ainda mais potente. Para examinar este comportamento diverso, foram feitas passagens seriadas em ovos embrionados em baixa e alta multiplicidade de infecção com estes vírus, sendo que esta última propicia a formação de partículas DI. Após cada passagem, o interferon humano de membrana amniótica (IFN-MA) foi induzido e foram determinados os títulos hemaglutinante e infeccioso dos vírus e o do IFN-MA.

A amostra Cantell mostrou uma redução de 10.000 vezes em seu título infeccioso com as passagens em alta multiplicidade. Os títulos de IFN-MA produzidos foram cerca de 300 unidades por ml nas passagens em baixa multiplicidade. Contudo, a capacidade indutora desta amostra subiu de 1.200 para 4.200 unidades por ml nas passagens com alta multiplicidade. Com a amostra Mill Hill, as passagens seriadas em baixa ou alta multiplicidade de infecção não modificaram os títulos infectantes ou as quantidades geradas de IFN-MA (cerca de 12.000 unidades por ml). Partículas "standard" e DI são necessárias para otimizar a produção de IFN-MA pela amostra Cantell. Com a amostra Mill Hill, todavia, não pode ser demonstrada a formação de partículas DI, nem modificações dos títulos de IFN-MA foram observados com a adição de partículas DI da amostra Cantell.

Palavras-chave: Interferon/Amniótica/partículas DI humano/paramixovirus.

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ANTAGONISM EXERTED BY AN ASSOCIATION OF STRICT ANAEROBIC BACTERIA FROM HUMAN FECAL FLORA AGAINST *CLOSTRIDIUM PERFRINGENS* IN GNOTOBIOTIC MICE

Jacques Robert Nicoli[1]
Pierre Raibaud[2]

SUMMARY

The antagonism between a limited number of strictly anaerobic bacteria isolated from human faecal flora and *Clostridium perfringens* type A (CpA) was studied in the intestinal tract of gnotobiotic mice. Association of 18 bacteria (three *Bacteroides*, five *Peptostreptococcus*, five *Eubacterium*, and five *Clostridium*) eliminated CpA from the intestines of all gnotobiotic mice in about three days. In the course of the simplification of this association from 18 to seven bacteria there was a loss in the capability of elimination of the target strain. Definitive elimination of CpA was observed in only 23% of gnotobiotic mice inoculated intragastrically with the seven barrier bacteria. These results suggest a complex relationship between bacteria directly involved in the antagonistic effect with helper strains.

Key words: Antagonism, *Clostridium perfringens*, Strict Anaerobic Bacteria.

INTRODUCTION

The barrier effect exerted by gastrointestinal microflora is one of the basic mechanisms that protect the host against enteric infections. Many authors have sought to identify the bacteria of the microflora responsible for such an antagonistic effect and to elucidate the mechanism of their action. This barrier effect is generally exerted by the association of a few different bacteria, individually inactive, and more rarely by a single one (1, 3, 4, 7). Bacteria responsible for a barrier effect against *Clostridium perfringens* have already been isolated from mice (3), piglets (7), and human (4).

The purpose of this study was to determine if there is an antagonistic effect of other human intestinal bacteria than *Peptostreptococcus* against *C. perfringens* and to isolate these microorganisms using gnotobiotic mice.

MATERIAL AND METHODS

Animals and diet - Adult axenic C3H mice were reared in Trexler-type isolators fitted with a rapid transfer system (La Calhéne, Vélizy-Villacoublay, France). They were fed a commercial diet) U. A. R., Epinay-sur-Orge, France) sterilized by *gamma* irradiation (40 kGy).

Bacterial strains - Strain CpA was a variant of a *Clostridium perfringens* strain belonging to serotype A which was unable to form thermotol-

1. Correspondence: Dr. Jacques Robert Nicoli, Departamento de Microbiologia, Instituto de Ciências Biológicas da UFMG, Caixa Postal 2486, 30161-960, Belo Horizonte, MG, Brasil.
2. Laboratoire d'Ecologie et de Physiologie du Tube Digestif, Institut National de la Recherche Agronomique, 78350, Jouy-en-Josas, France.

erant spores in the intestines and was devoided of lecithinase. Spores of a thermophilic *Bacillus subtilis* strain were used as transit marker, as described previously (7). Antagonistic bacteria against CpA were obtained from freshly collected human feces which were introduced into an anaerobic chamber (La Calhéne, Vélizy-Villacoublay, France). The preweighed sample of these feces was homogenized with an Ultraturrax (O. S. I., Paris, France) in diluting medium LCY (5), and 10-fold dilutions were made. Aliquots of 0.1 mL were planted on Freter medium and brain heart infusion agar medium and incubated anaerobically for seven days at 37°C in the anaerobic chamber. One hundred and twenty colonies were picked at random from dominant flora (Petri dishes with 10^{-8} and 10^{-9} dilutions) and subcultured on brain heart infusion agar until heavy growth was obtained. The isolated bacteria were identified by their morphocellular characteristics on the media and by their biochemical and physiological properties (2).

Inoculation of non-pathogenic CpA in human - A 5-mL suspension (10^8 viable cells/ml) of the non-pathogenic CpA mutant was administered orally to an human adult volunteer simultaneously with a 5-mL spore suspension (10^8 spores/ml) of the transit marker. Feces were analyzed daily for CpA and thermophilic *B. subtilis*.

Association of axenic mice with bacterial strains - Target strain (CpA) was grown in soft medium W (7). A suspension containing 10^8 vegetative cells/ml of the CpA inoculum was mixed (CpA + TM) or not (CpA) with 10^8 spores/ml of the transit marker. Antagonistic strains, isolated as described above, were grown on brain heart infusion agar (Difco Laboratories, Detroit, MI, U.S.A.) for 48 hrs at 37°C in the anaerobic chamber. Then, they were washed off with LCY diluent, mixed and the resulting suspension sealed anaerobically and introduced in the isolator. All inocula (CpA, CpA + TM or the different suspension of antagonistic mixed bacteria: 120B, 18B and 7B) were administrated orogastrically with 1 ml for each axenic mouse.

Bacterial counts - Freshly collected feces were diluted 100-fold in LCY diluent and homogenized by hand. A 1-mL amount of adequate serial 10-fold dilutions mixed with 14 mL of solid W medium supplemented with 0.013% neomycin was poured into 8 x 400 mm tube (Touzard & Matignon, Vitry-sur-Seine, France). Incubation was done at 37°C. One day later, colonies of CpA were counted. Spores of the transit marker were counted by plating on MS medium (4). Incubation was done aerobically at 55°C for 24h.

RESULTS AND DISCUSSION

Although, *C. perfringens* is not generally allowed to multiply in the human gut, it occurs occasionally in numbers of about 10^4 cells per gram of wet feces in the large intestine of some healthy man (6), probably as a result of a partial barrier. Search of *C. perfringens* in the feces of our volunteer was unsuccessful, suggesting the presence of a drastic barrier. This hypothesis was confirmed by the elimination of the non-pathogenic CpA when inoculated orally in this volunteer (Fig. 1). Comparison of the rate of fecal eliminitation of CpA and of the transit marker shows that the CpA inoculum (5 x 10^8 cells) was eliminated faster than the transit marker inoculum (5 x 10^8 cells). The lower initial level of the target strain in the feces suggests a bactericidal process due to microbiological or acidic gastric barrier.

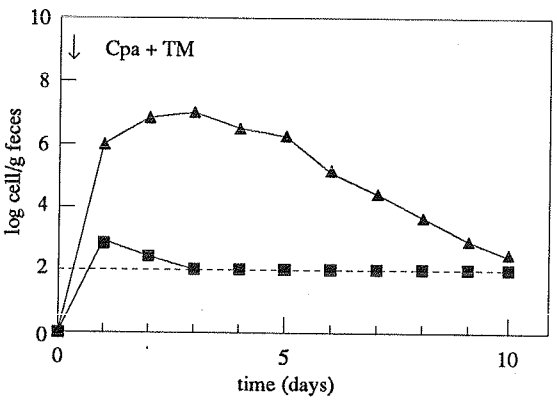

FIGURE 1 - Antagonistic effect of intestinal flora against *Clostridium perfringens* (■) in human. Transit marker (▲). Arrow shows the inoculation time of the human volunteer with *C. perfringens* and the transit marker (Cpa + TM).

The inoculation of 120 strict anaerobic dominant bacteria from volunteer feces in mice monoassociated with the target strain led to an elimination of the later in about two days (Fig. 2). In a second challenge with the target bacteria and the transit marker, an elimination of bacterial cells and spores similar to that of Figure 1 was observed. Search for inhibitory diffusible substance for *C. perfringens* in feces of the gnotobiotic mice was unsuccessful. CpA levels were

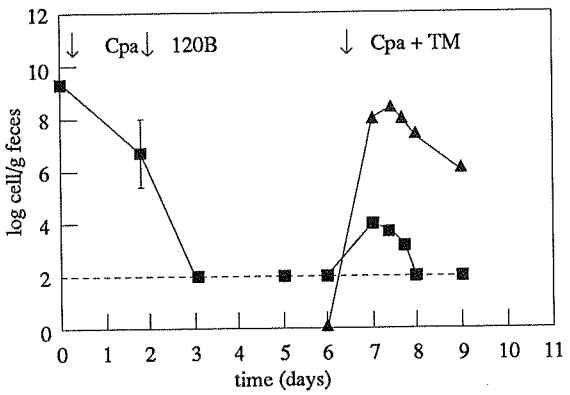

FIGURE 2 - Antagonistic effect of bacteria from human intestinal flora against *Clostridium perfringens* A (■) in mice. Transit marker (▲). Arrows show the inoculation times of *C. perfringens* (Cpa), the 120 bacteria (120B), and *C. perfringens* and the transit marker (Cpa + TM).

lower than those of the transit marker as in human feces, but on the other hand, elimination times were smaller both for CpA cells and marker spores.

Based on morphological and culture characteristics we have observed that these 120 bacteria were constituted of 18 really different bacteria and their numerous repetitions. These 18 selected bacteria represented the main genera of the human normal intestinal flora, namely 3 *Bacteroides*, 5 *Eubacterium*, 5 *Peptostreptococcus*, and 5 *Clostridium*. Ten of these strains were extremely oxygen sensitive (EOS) (4 *Peptostreptococcus*, 4 *Eubacterium*, and 2 *Clostridium*). Figure 3 shows the drastic antagonistic effect against CpA of these 18

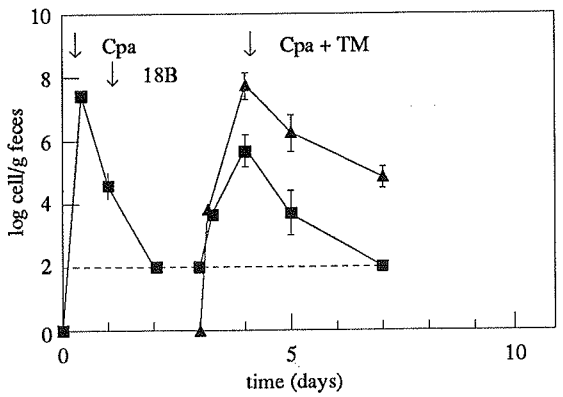

FIGURE 3 - Antagonistic effect of 18 bacteria from human intestinal flora against *Clostridium perfringens* A (■) in mice. Transit marker (▲). Arrows shown the inoculation times of *C. perfringens* (Cpa), the 18 bacteria (18B), and *C. perfringens* and the transit marker (Cpa + TM).

bacteria when inoculated in eight mice monoassociated with the target strain. CpA was eliminated in about four days both after the first or the second challenge in all animals.

Using several different combinations of the 18 bacteria we could reduce the number of barrier component to seven. The seven bacteria were constituted of 2 *Bacteroides*, 3 *Peptostreptococcus* (1 EOS), and 2 *Eubacterium* (1 EOS). However, during the simplification process of the barrier association against CpA we have noted a loss in the installation efficiency of the antagonistic effect in gnotobiotic mice. Thirteen mice associated with the seven bacteria were inoculated with CpA (Fig. 4). A drastic decrease in number of CpA was observed in all animals and the target strain reached a level below 100 viable cells per gram of wet feces within two or three days. However, ten mice failed to eliminate CpA definitively. In these mice a posterior increase in the target strain population was observed and a stable level of 10^4 - 10^5 CpA cells per gram of wet feces was reached in about nine days. The reason of this individual variation between inbred mice could not be explained. Different population levels of some barrier bacteria could be responsible for this discrepancy. Indeed, after inoculation of axenic mice with diluted feces obtained from a mouse unable to eliminate CpA, a drastic barrier against CpA was observed in the former (data not shown).

Therefore, the human barrier against CpA can be much more complex than those obtained from mice (3 *Clostridium* sp) (3) or piglets (*Bacteroides thetaiotaomicron* and *Fusobacterium necrogenes*) (7). Our results also illustrate the presence of redundant barrier mechanisms in the digestive tract. In effect, a *Peptostreptococcus* from human fecal flora have the same barrier effect against *C. perfringens* A in gnotobiotic mice (4). The advantage of such redundancy of protective mechanisms in terms of host survival is obvious. However, the mechanism of bacterial antagonism must be different since, contrarly to the *Peptostreptococcus*, the barrier effect described here does not involve any mediation through a soluble substance, but probably a direct contact between the bacterial cells responsible for this barrier effect and the bacterial target. Studies on the effect of inoculation of the 18-barrier bacteria in humans are in course. The success of these studies will be very significant, since it represents an ecological therapy replacing the current antibiotic therapy.

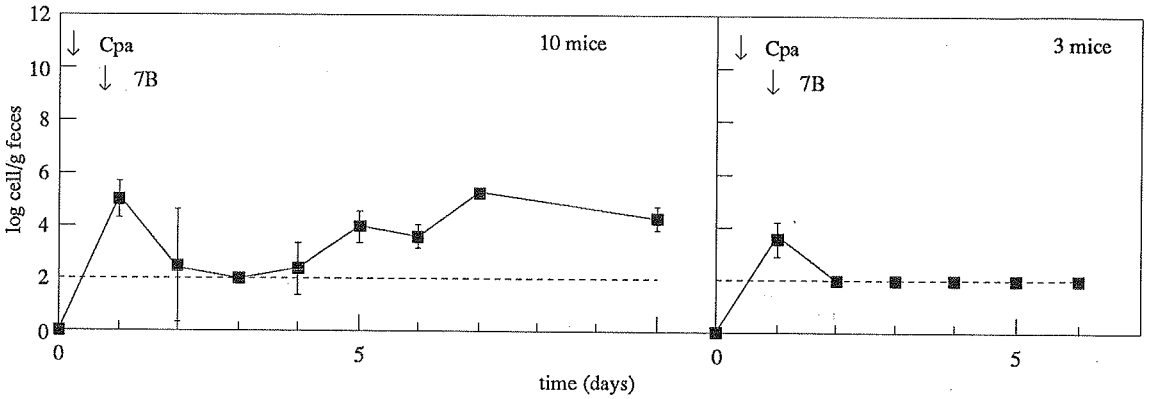

FIGURE 4 - Antagonistic effect of seven bacteria from human intestinal flora against *Clostridium perfringens* A in mice. Arrows show the inoculation times of *C. perfringens* (Cpa) and seven bacteria (7B).

RESUMO

Antagonismo exercido por bactérias anaeróbias estritas da flora fecal humana contra *Clostridium perfringens*, estudado em camundongos gnotobióticos.

O antagonismo entre um número limitado de bactérias anaeróbicas estritas isoladas de uma flora fecal humana e *Clostridium perfringens* tipo A (CpA) foi estudado no trato intestinal de camundongos gnotobióticos. Uma associação de 18 bactérias (três *Bacteroides*, cinco *Peptostreptococcus*, cinco *Eubacterium* e cinco *Clostridium*) eliminou CpA dos intestinos de todos os camundongos gnotobióticos em três dias. Durante o processo de simplificação dessa associação de 18 para sete bactérias houve uma perda na capacidade de eliminar a cepa alvo. Uma eliminação definitiva de CpA foi observada em somente 23% dos camundongos inoculados intragastricamente com as sete bactérias. Esses resultados sugerem uma relação complexa entre as bactérias diretamente envolvidas no efeito antagonista com cepas auxiliares.

Palavras-Chave: Antagonismo, *Clostridium perfringens*, Bactérias anaeróbicas estritas.

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SALMONELLA SEROTYPES FROM EFFLUENT SEWAGE WATERS: LEVELS OF RESISTANCE TO HEAVY METALS, AND MARKERS TRANSFER

Roberta Salgado Pinto Nogueira
José Cavalcante de Albuquerque Ribeiro Dias
Ernesto Hofer

SUMMARY

A total of 278 *Salmonella* strains resistant to the heavy metals Cu, Hg and Zn were analyzed. The strains included the following four serotypes which were prevalent in isolates from the effluents of two sewage treatment plants in the city of Rio de Janeiro, RJ, from Brazil, 1984 to 1985: *S. panama* (118), *S. typhimurium* (58), *S. agona* (53) and *S. oranienburg* (49).

Confirmation of the resistance patterns using a resistogram indicated *in vitro* stability for 96.7% of the sample, including the Cu and Hg markers and excluding Zn.

Up to 68.7% of the strains were resistant to 200 ug/ml Cu and 77.7% were resistant to 45 ug/ml Hg. These frequencies were obtained by submitting the *Salmonella* strains to increasing concentrations of Cu (100 to 250 ug/ml) and Hg (5 to 50 ug/ml) incorporated into nutrient agar.

For a total of 268 conjugation experiments, in which the standard *Escherichia coli* K 12 F^-Nal^r strain and cultures isolated from sewage water (*Citrobacter freundii* Tc^r and *Salmonella oranienburg* Sm^r) were considered to be the recipients, Cu^r transconjugants were isolated at an overall rate of 19.3% and Hg^r transconjugant at a rate of 86.4%. All of the transconjugant analyzed expressed the same level of resistance as the corresponding *Salmonella* (donor) strain.

We discuss the significance of these results in terms of the dissemination of genetic markers in view of the continued discharge of sewage effluent contaning these microorganisms into the Bay of Guanabara.

Key words: *Salmonella*, sewage water, heavy metals, plasmids.

INTRODUCTION

The growing and often disorganized construction of industrial complexes in the urban and periurban areas of large cities over the last few decades has led to critical and undesirable ecological situations, especially when industrial refuse is discharged into river and bay waters without appropriate treatment (16). Among the contaminants, heavy metals are a matter of particular concern in view of their action also at the level of either autochthonous or transitory microorganisms present in the effected bodies of water which may lead to the emergence of bacterial

Departamento de Bacteriologia - Instituto Oswaldo Cruz - FIOCRUZ - Avenida Brasil, 4365 - Manguinhos - 21040-360 - Rio de Janeiro - RJ - Brasil

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strains resistant to metal ions through a selective process (5,7).

Another facet of the problem is the elimination of bacteria bearing genetic markers, *e. g.*, resistance to heavy metals, including pathogens such as *Salmonella* that reach the different water bodies involved (bay waters, for example) through the effluents of sewage treatment plants (4). Thus, even though the purifying ability of sea water is known, the survival of bacteria such as *Salmonella* depends, among other factors, on the biotic and abiotic conditions of these waters. Different studies have pointed out the occurrence of *Salmonella* in sea water and emphasized the role of this incidence in the epidemiology of enteric infections (10,17).

In view of the above considerations, we undertook a study on *Salmonella* strains resistant to the heavy metals cooper, mercury and zinc and belonging to four serotypes prevalent in the effluents of sewage treatment plants in the city of Rio de Janeiro, RJ, Brazil, in order to determine their maximum level of resitance to the metals, their ability to transfer this resistance to other enterobacteria, and the level of resistance in the transconjugant strains detected.

MATERIAL AND METHODS

Sample: 278 *Salmonella* strains resistant to heavy metals were selected from the Culture Collection of the National Reference Center for *Salmonella* of the Department of Bacteriology, Instituto Oswaldo Cruz - FIOCRUZ.

The cultures (Table 1) were isolated from the effluents of the sewage treatment plants of Penha (Estação de Tratamento de Esgotos da Penha - ETEP) and of Ilha do Governador (Estação de Tratamento de Esgotos da Ilha do Governador - ETIG) in the city of Rio de Janeiro from 1984 to 1985. They were distributed among four serotypes prevalent at both sources, i. e., *S. panama* (118), *S. typhimurium* (58), *S. agona* (53) and *S. oranienburg* (49), previously described by Aguas (1). The collection cultures were maintained in tubes containing phosphate agar without the addition of heavy metals and sealed with paraffin-covered corks, which were stored at room temperature for aproximately 2 years until the time for analysis.

Resistance to the heavy metals cooper, mercury and zinc has been previously characterized and published in partial by Dias et al. (4).

Resistogram: to confirm the patterns of resistance to heavy metals, all strains were submitted to a second resistogram using the agar dilution method (7).

The cultures were grown in Nutrient Broth (Merck) and plated onto Petri dishes containing aqueous solutions of the metals at the following concentrations: cooper sulfate (Cu) - Mast, 100 ug/ml; mercuric chloride (Hg) - Baker & Adamson, 5 ug/ml, and zinc sulfate (Zn) - Baker Analysed Reagent, 100 ug/ml. *Escherichia coli* ATCC 25922, which is sensitive to the metals, was used as control.

Determination of Maximum Levels of Resistance to Heavy Metals: Salmonella strains were submitted to the minimum inhibitory concentration (MIC) test for Cu (272) and Hg (18) - Table 2 using the procedures cited above. Concentrations of 100, 150, 200, and 250 ug/ml were for Cu and concentrations of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 ug/ml for Hg. The inoculated plates were incubated at 37°C. Maximum resistance level was defined as the highest metal concentration at which bacterial growth was observed, i. e. the concentration immediately preceding the MIC.

Conjugation experiments: to investigate the transfer of Cu resistance markers in a larger number of *Salmonella* strains, 98 cultures were selected, 80 of which were resistant to Cu (most of them to as much as 200 ug/ml), as well as all of the 18 cultures resistant to Hg, regardless of the maximum level of resistance observed. Cultures in which a change in the original resistance pattern was observed were not considered in this test (Table 2).

The receptors used were the standard *Escherichia coli* K 12^{-} Nal^{r} strain (Laboratório de Fisiologia Celular, Instituto de Biofísica, Universidade Federal do Rio de Janeiro), cultures of *Citrobacter freundii* AIX 21 resistant to tetracycline (Tc) isolated from ETIG effluent (9) and of *Salmonella oranienburg* SEI 04 resistant to streptomycin (Sm) isolated from ETIG effluent (1).

The conjugant mixtures were incubated in a double boiler at 37°C for 24 h and plated onto nutrient agar (20) with Cu (200 ug/ml) of Hg (5 ug/ml) added. The antimicrobial agents nalidixic acid (Nal) - Sidney Ross Co., 100 ug/ml, tetracycline (Tc) - Laborterápica Bristol, 20 ug/ml or streptomycin (Sm) - Fontoura Wyeth S.A., 10 ug/ml were incorporated for strain counter selection according to the receptor strain used. In the testes of Hg marker transfer, the *S. typhimurium* strains were not conjugated with the receptor strains from sewage water because they presented the same pattern of antimicrobial resistance.

After incubation at 37°C for 24 - 48 h, approximately 5 transconjugant colonies per experiment were submitted to biochemical species confirmation and to the determination of the level of resistance to the heavy metal by plating the samples onto nutrient agar plus a solution of the metal (Cu or Hg) at a concentration identical to the previously defined as maximum for the corresponding donor strains.

TABLE 1 - Numerical sample distribution according to origin and serovars*

Origin	Serovars				Total
	S. agona	*S. oranienburg*	*S. panama*	*S. typhimurium*	
ETEP	33	28	68	30	159
ETIG	20	21	50	28	119
Total	53	49	118	58	278

* Águas (1987)

RESULTS AND DISCUSSION

The first approach concerns the *in vitro* stability of the markers of resistance to heavy metals in the *Salmonella* strains. Remember what the collection were not maintained in medium with metals. Thus, the fact that a percentage of 96.7 (Table 2) was detected for the confirmation of the resistance patterns permits us to assume that the survival of these strain *in natura* under selective pressure by metal ions is insured. It should be pointed out that the final destination of the effluents from the two treatment plants (ETEP and ETIG) from which the strains were obtained is the Bay of Guanabara, Rio de Janeiro, RJ, in which elevated concentrations of heavy metals have been detected (16).

On the other hand, the loss of the Hg and Zn markers was observed, with Zn being detected in a single strain of *S. typhimurium*, whereas the Cu^r phenotype persisted (Table 2). Marker stability was not identical for the four serotypes and of the nine cultures which revealed a change in the original resistance pattern, five were *S. typhimurium*, two *S. panama* and one *S. agona*. No loss of resistance determinants was detected in the *S. oranienburg* serotype.

Resistance to Cu was 68.7% up to concentrations of 200 ug/ml, and resistance to Hg was 77.7% at the concentration of 45 ug/ml. It can be seen that most *Salmonella* strains were resistant to concetrations as high as 200 ug/ml, the only different ones being those of the *S. agona* serotype, 92.3% of which had a lower resistance level (Table 3). As to Hg, the strains were particularly resistance to the 45 ug/ml concentration, with maximum percentages being obtained for the *S. panama* and *S. agona* serotypes. Considered as a whole, the *Salmonella* serotypes revealed a certains homogeneity in phenotypic response to different heavy concentrations. Indeed, this result extends data reported by Solari (18) in a study of epidemiologic marker tracking in *S. agona* from different sources, in which the author did not attribute a high degree of heterogeneity to the resistogram.

TABLE 2 - *In vitro* stability of resistance to heavy metals in the 278 *Salmonella* strains.

Original Pattern	Nº	Confirmed Pattern	Nº	%	Confirmed Phenotype Cu^r	Hg^r	Zn^r
Cu	251	Cu	251	100	251	–	–
Hg	6	Hg	1	16.6	–	1	–
Cu-Hg	20	Cu-Hg	17	85.0	20	17	–
Zn	1	–	0	–	1	–	0
Total	278		269	96.7	272	18	0

With respect to comparison of the results related to resistance to heavy metals with those obtained by other authors, Carneiro et al. (3) have pointed out the effects of limiting factors such a pH, culture medium and inoculum. Nevertheless, even considering these variables and the use of different bacterial models, there are similarities and differences worth discussing. Kesin & Karasyova (12), for example, observed resistance to as much as 80 ug/ml Hg in bacteria from areas contaminated with this metal, whereas Avila Campos et al. (2), in a study of resistance to mercury chloride in *Actinobacillus actinomycetemcomitans* of human and environmental origin using MIC, detected absolute sensitivity to the 4 ug/ml concentration. In contrast, Dias et al. (6), in a study on *Escherichia coli* isolated from sewage water, i. e., the same source as for the strains investigated in the study, detected resistance to as much as 300 ug/ml Cu and 30 ug/ml Hg.

In the conjugation experiments (Table 4 and 5) particularly noteworthy was the fact that the *Citrobacter freundii* Tc^r and *Salmonella oranienburg* Sm^r cultures did not transfer their respective markers, Tc and Sm, to *E. coli*. This result was of great help for the use of these strain in the transfer tests, as explained in detail further on.

TABLE 3 - Levels of resistance to Cu and Hg in *Salmonella* serovars.

Serovars	Nº of cultures Cu^r	Copper (ug/ml) 100	150	200	250	Nº of cultures Hg^r	Mercury (ug/ml) 5 - 25	30	35	40	45	50
S. panama n = 118	118	6	19	93 (78.8)**	–	1	→			→	1 (100)	–
S.typhimurium n = 58	53	→	3	50 (86.2)	–	6	→	1	1	→	4 (66.6)	–
S. agona n = 53	52	2	48 (92.3)	2 (3.7)	–	2	→			→	2 (100)	–
S. oranienburg n = 49	49	→	7	42 (85.7)	–	9	→	1	1	→	7 (77.7)	–
Total	272	8	77	187 (68.7)	–	18	→	2	2	→	14 (77.7)	–

* No. of cultures analysed.
** The numbers in parentheses are percentages.

Table 4 reveals that transfer of the Cu marker was of the order of 19.3% for a total of 129 experiments and was mainly related to the *S. panama* serotypes (31.1%) for the receptor strains *C. freundii* and *S. oranienburg*, followed by transfer of *S. agona* (5.5%) and *S. oranienburg* (16.6%). Cu^r transconjugants were never isolated from the 20 *S. typhimurium* strains when the latter were used as "donors".

TABLE 4 - Transfer of the Cu Markers in the *Salmonella* serovars.

Donor cultures		Receptor cultures			Total
Serovars	Nº	*Escherichia coli*	*Citrobacter freundii*	*S. oranienburg*	
S. panama	30	0/30*	6/13 (46.1)**	13/18 (72.2)	19/61 (31.1)
S. typhimurium	20	0/8	0/6	0/6	0/20 (0.0)
S. agona	12	0/6	1/6 (16.6)	0/6	1/18 (5.5)
S. oranienburg	18	0/12	2/7 (28.5)	3/11 (27.2)	5/30 (16.6)
Total	80	0/56	9/32 (28.1)	16/41 (39.0)	25/129 (19.3)

* Number of effective conjugations/number of conjugations performed.
** The numbers in parentheses are percentages.

Among the receptor cultures, *S. oranienburg* was the most effective in receiving the Cu markers, reaching a rate of 39.0% positive conjugations, possibly due to the greater phylogenetic affinity between *Salmonella* serotypes (19). On the other hand, no *Salmonella* strain transferred the Cu marker to the *E. coli* K 12 culture. In this case, the difference between bacterial special is an unlikely explanation for this result since the Hg marker detected in these samples was transferred to the standard culture (Table 5) and the *C. freundii* strain received the Cu marker at a rate 28.1% (Table 4). A genetic distinction may exist between the markers that confer resistance to Cu, since Vicente et al. (20) obtained a discrete frequency of Cu^r transconjugants for *E. coli* strains resistant to copper sulfate isolated from sewage. However, Lima and Silva (13) pointed out the 100% transfer rate of *E. coli* strains from fish catches that were conjugated with the *E. coli* K 12 strain mentioned above.

Transfer of the Hg marker reached a rate of 84.6% for the experiments as a whole (Table 5).

TABLE 5 - Transfer of the Hg Markers in the *Salmonella* serovars.

Donor cultures		Receptor cultures			Total
Serovars	Nº	*Escherichia coli*	*Citrobacter freundii*	*S. oranienburg*	
S. panama	1	1/1* (100)**	0/1	1/1 (100)	2/3 (66.6)
S. typhimurium	6	6/6 (100)	0/0	0/0	6/6 (100)
S. agona	2	2/2 (100)	0/2	2/2 (100)	4/6 (66.6)
S. oranienburg	9	8/9 (88.8)	7/7 (100)	6/8 (75.0)	21/24 (87.5)
Total	18	17/18 (94.4)	7/10 (70.0)	9/11 (81.8)	33/39 (84.6)

* Number of effective conjugations/number of conjugations performed.
** The numbers in parentheses are percentages.

In contrast to the Cu marker, the resistance to Hg was almost fully transferred to the standard strain. Among the donor strains, a similar transfer to the various receptors was observed for *S. oranienburg*. In contrast, the *C. freundii* receptor was unable to receive the Hg marker of *S. panama* and *S. agona.*

The transfer of these markers takes on more importance when we consider that for the transconjugant cultures analyzed as a whole, the levels of resistance to the heavy metals Cu and Hg were equal to those expresses by the respective donor strains. As a consequence, the predominance of *S. typhimurium*, *S. agona* and *S. oranienburg* serotypes and the detection of *S. panama* reported by Rodrigues et al. (17) for different beaches along the Bay of Guanabara probably represents not only a correlation with the Salmonnella serotypes prevalent in sewage effluent discharged into the bay (1), but also the ability of this pathogen to adapt to the abiotic conditions of these waters (16), in the presence of Hg and Cu as the metals which significantly pollute the Bay of Guanabara. It is also believed that the halotolerance analyzed in part of the samples (4), reaching the concentration of 8.0 g% NaCl and comparable to that presented by a *Vibrio alginolyticus* ATCC 17749 standard (an autochthonous marine specie) may contribute to the survival and permanence of *Salmonella* in this biological system. Indeed, the viability in sea water of *Salmonella* serotypes from sewage water mentioned by Hofer & Costa (10) is additional evidence in support of this hypothesis.

This situation, in addition to representing deterioration of the health aspect, also has repercussions on marine microorganisms, e. g. strains of the genus *Vibrio*. Thus, the detection of *Vibrios* isolated from the intestinal tract of fish from the Bay with simultaneous resistance to antimicrobial agents and to Hg (5) indicates the possible dissemination of genetic markers of *Salmonella* (and/or of other enterobacteria) towards this authochtonous genus, since in eleven of the transconjugant cultures studied here, more than 90.0% co-transfer was detected in a previous study (15) for the determinants of resistance to sulfadiazine, streptomycin, tetracycline, kanamycin, chloramphenicol, ampicillin and/or gentamicin.

On this basis, additional lines of investigations should be followed to obtain greater efficiency of sewage treatment processes, minimizing the elimination of bacteria from effluents to the environment especially of pathogens such as *Salmonella* which harbor genetic factors.

RESUMO

Sorotipos de *Salmonella* de efluente de esgoto: níveis de resistência a metais pesados e transferência de marcadores.

Foram analisadas 278 amostras de *Salmonella* resistentes aos metais Cu, Hg e Zn, correspondendo os sorotipos: *S. panama* (118), *S. typhimurium* (58), *S. agona* (53) e *S. oranienburg* (49), prevalentes no isolamento a partir de efluentes de duas estações de tratamento de esgoto, no período de 1984 a 1985, na cidade do Rio de Janeiro, Brasil.

A confirmação dos perfis revelou a perda dos marcadores Hg e Zn, este último ocorrente em uma única amostra. No entanto, para a totalidade das amostras obteve-se 96,7% de estabilidade *in vitro* dos marcos Cu e Hg, prevendo-se uma maior sobrevivência dessas amostras de *Salmonella* em ambientes onde tais metais exerçam pressão seletiva.

Para a determinação de resistência aos metais utilizou-se o método de diluição em placa contendo Ágar Nutriente (Merck), incorporando-se concentrações crescentes de sulfato de cobre (Mast): 100, 150, 200 e 250 ug/ml e de cloreto de mercúrio (Baker & Adamson): 5, 10, 15, 20, 25, 30, 35, 40, 45 e 50 ug/ml. Definiu-se como nível máximo de resistência a concentração mais elevada do metal onde se observou crescimento bacteriano. Deste modo, para o Cu, 68,7% das amostras foram resistentes até 200 ug/ml e para o Hg, até 45 ug/ml, 77,7%. Apenas *S. agona* (92,3%) apresentou resistência ao nível de 150 ug/ml de Cu, enquanto *S. panama* (78,8%), *S. typhimurium* (86,2%) e *S. oranienburg* (85,7%) exprimiram até 200 ug/ml. Quanto ao Hg, a maioria das amostras (77,7%), independente do sorotipo, revelou-se resistente até a concentração de 45 ug/ml. No conjunto, houve certa homogeneidade no comportamento dos sorotipos de *Salmonella* às distintas concentrações dos metais.

Nos experimentos de conjugação verificou-se a capacidade de transferência dos marcadores albergados nas amostras, para outras enterobactérias. Além da receptora padrão, *Escherichia coli* K 12 F^- Nal^r, as culturas de *Citrobacter freundii* Tc^r e de *Salmonella oranienburg* Sm^r isoladas de águas de esgoto, incapazes de transferirem os marcos Tc e Sm para a cepa padrão de *E. coli*, foram utilizadas.

Para o Cu foram realizados 129 testes, compreendendo 80 amostras de *Salmonella* com resistência, em sua maioria, até 200 ug/ml. Para o Hg,

tomou-se a totalidade de amostras (18), resultando 39 testes. Transconjugantes Cu^r foram obtidos na taxa de 19,3% e para Hg^r, em 84,6%. Dentre as culturas receptoras, *S. oranienburg* mostrou-se mais efetiva na recepção do marco Cu. Nenhuma das amostras foi capaz de transferir o marco Cu para a *E. coli* K 12. Assinala-se ainda que entre as culturas doadoras, *S. panama* revelou o maior percentual de conjugação (31,1). Nas 20 amostras de *S. typhimurium* não foram isolados transconjugantes Cu^r. A resistência ao Hg foi transferida para a *E. coli* K 12 em 94,4%. As amostras de *S. oranienburg* transferiram este marcador de modo equitativo: 88,8% para *E. coli* K 12; 100% para *C. freundii* e 75,0% para *S. oranienburg*. A totalidade das culturas transconjugantes apresentou níveis de resistência aos metais pesados iguais àqueles expressos pelas amostras doadoras correspondentes.

Os metais Cu e Hg são apontados pela bibliografia como os mais significativos na poluição da baía de Guanabara. Assim, postula-se sobre a sobrevivência e permanência de *Salmonella* lançada por efluente neste bioma, visto o isolamento dos sorotipos de *S. agona*, *S. typhimurium*, *S. oranienburg* e *S. panama* entre aqueles prevalentes ou detectado em águas de praias dessa baía, citado por outros autores.

Palavras-chave: *Salmonella*, águas de esgoto, metais pesados, plasmídeos.

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VIBRIO PARAHAEMOLYTICUS IN LOBSTER *PANULIRUS LAEVICAUDA* (LATREILLE)

Regine H. S. F. Vieira[1]
Sebastião Timo Iaria[2]

SUMMARY

This study verifies the presence of *Vibrio parahaemolyticus* on 16 tails samples of the lobster *Panulirus laevicauda* (Latreille) species collected at the Fish Market in 1988, on 16 samples collected at the same location in 1990 and on 16 samples collected at a local Fish Industry. The MPN of *V. parahaemolyticus* obtained on samples from all sources varied from < 3.0 to 21.0/g. of 39 strains identified as *V. parahaemolyticus* on the Fish Market samples collected in 1988, 11 (28.2%) showed to be "non agglutinable" and among isolates of 1990, 5 (35.7%) of them agglutinated with anti-K sera. Only one isolate among Industry samples was typable. The serogroups found among *V. parahaemolyticus* strain were: KI, KII, KIII, KIV, KV, KVI, KVII, KVIII and KIX. From a total of 56 *V. parahaemolyticus* strains isolated on Fish Market samples collected in 1988 and 1990, 29 (51.8%) were Kanagawa-positive, whereas those isolated on samples the from Industry (3) were Kanagawa-negative.

Key words: *Vibrio parahaemolyticus;* lobster; Kanagawa test.

INTRODUCTION

Many research works related to *Vibrio parahaemolyticus* have been reported since its isolation from "shirasu" in Japan (Fujino, 1953). Most of these studies report the occurrence of this bacterium in sea water and seafood such as crabs, shrimps, oysters, fish and, rarely, lobsters (Miyamoto and Kuroda, 1975; Seng and Jegathesan, 1977; Gelli et al., 1979; Hofer and Silva, 1986; Khyukia et al., 1989). Outbreaks detected in different countries among seafood consumers have been studied by Molenda et al. (1972), Regasens et al. (1974), Buogo et al. (1979), and Karunasagar and Mohankumar (1980).

The action of this bacterium on the human organism has been studied mainly by Boutin et al. (1979), Hackney et al. (1980a), and Merrel et al. (1983). The main reason for the severe gastroenteritis resulting from its action is still being discussed.

Many researchers mention the Kanagawa factor as partially responsible for enteropathogenesis of this bacterium to man (Miyamoto et al., 1969; Zen-Yoji et al., 1971). It is still accepted that strains obtained from clinical cases are Kanagawa-positive and those isolated from food are negative to test (Sakazaki et al., 1968; Furniss et al., 1978).

1. Departamento de Engenharia de Pesca, Laboratório de Ciências do Mar. UFC. Av. da Abolição, 3027 - CEP 60165 - Fortaleza - Ceará - Brasil.
2. Departamento de Microbiologia - Instituto de Ciências Biomédicas - Universidade de São Paulo - Av. Lineu Prestes, 1374 - CEP 05508 - São Paulo - Brasil.

The State of Ceara (Brazil) is the largest lobster exporter Brazilian state, and yielded an average production of aproximately 2110 Tons/year of lobster tails over the last 10 years. The quality control is limited to those being exported leaving the city population exposed to the dangers of consuming crustaceans which have not been approved by the processors, and have therefore been sold in the permanent Fish Market at Mucuripe.

The objectives of this study were: 1) to determine the level of *V. parahaemolyticus* contamination in lobster tails samples from Mucuripe Fish Market (Fortaleza, Ceara) collected in 1988 and 1990 through the "Most Probable Number" test (MPN) using samples taken at the processing industry in Fortaleza as control: and 2) to determine Kanagawa-phenomenon in these isolates.

MATERIALS AND METHODS

The samples consisted of three sets of 16 samples, each one weighing 500 g and containing 5 - 9 lobster (*Panulirus laevicauda*) tails taken at Mucuripe Fish Market in 1988 and 1990 and in one of the fish processing industries in Fortaleza, Ceara State, Brazil in 1988.

The samples, still in plastic bags used by the fishmongers, were transported from the Fish Market and from the industry to the laboratory in cool boxes. Tests were conducted within an hour of the samples arrival at the laboratory.

The quantification of *V. parahaemolyticus* in lobster tail samples was made through the MPN test. The identification tests were carried out according to Twedt (1984).

The "Denka-Seiken K. K." anti-K sera kit was used to classify the *V. parahaemolyticus* strains into serogroups, according to Twedt (1984).

The Kanagawa test was applied to the strains according to ICMSF (1978).

A Statistic Proportions Test (Mendenhall, 1983) was used to compare the 1988 versus 1990 samples in terms of positivity to both the *V. parahaemolyticus* identification test and the Kanagawa test.

RESULTS AND DISCUSSION

Table 1 and Figure 1 show that the ranges of *V. parahaemolyticus* per gram varied only slightly between samples: < 3,0 to 21,0 on samples from the Fish Market collected in 1988; < 3,0 to 6,1 on the collected samples in 1990; and < 3,0 to 4,0 on samples from the processing industry, showing quantitatively a very low contamination level by this bacterium. In fact, none of the analyzed samples exceeded the limit set by Regulation 01/87 from National Division of Sanitary Surveillance (Brazilian Ministry of Health, National Secretary of Sanitary Surveillance), which allows a maximum of 10^3 *V. parahaemolyticus* per gram in chilled or frozen raw crustaceans.

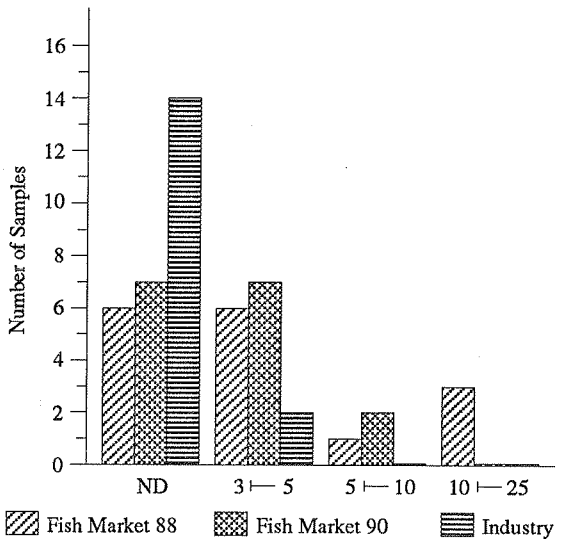
Karanusagar et al. (1984), quantifying *Vibrio parahaemolyticus* in frozen, cooked and raw shrimps obtained from a processing plant located near Mangalore, India, found that most of the analyzed samples showed a MPN of less than 10/g and none has exceeded 10^2/g. According to them the low numbers of this bacterium found in frozen and chilled raw shrimps can be explained by the low concentration of *V. parahaemolyticus* in the marine environment by the reduction of the number of the bacterium through the chilling or freezing process or both. For the cooked shrimps, the MPN levels were 10 to 10^2/g, and can be explained by cross-contamination.

There were grounds for anticipating high values of MPN of *V. parahaemolyticus* in the present study. Temperatures at the collection site are high and constant throughout the year. In ad-

TABLE 1 - Most Probable Number (MPN/g) of *Vibrio parahaemolyticus* distributed by class intervals in 48 lobster tails samples obtained in the permanent Fish Market at Mucuripe in 1988 and 1990 from the fish industry, Fortaleza, Ceara, Brazil.

Source	Number of samples	ND*	Number of samples				
			3 ⊢ 5 MPN/g	5 ⊢ 10 MPN/g	10 ⊢ 23 MPN/g	Nº	%
Fish Market 1988	16	6	6	1	3	10	62.5
Fish Market 1990	16	7	7	2	–	9	56.2
Industry	16	14	2	–	–	2	12.5

* None *V. parahaemolyticus* in any examined portion.



* ND - None *V. parahaemolyticus* in any examined portion.

FIGURE 1 - Most Probable Number (MPN/g) of *Vibrio parahaemolyticus* distributed by class intervals in 48 lobster tail samples obtained from the Permanent Fish Market at Mucuripe in 1988 and 1990 and from a local Fish industry, Fortaleza, Ceara, Brazil.

dition, all the analyzed lobsters have been stored on ice for several days prior to collection. With the exception of those from the processing industry, all showed signs of decomposition, including a noticeable odor, at the time of collection. Furthermore, the studied lobster species P. laevicauda is found in large quantities in coastal waters, and its microbiota is very rich (Vieira, 1985). In spite of this, the low MPN found in the present study can be related to *P. laevicauda's* habitat in the open sea, where *V. parahaemolyticus* can be present in reduced numbers (Kaneko and Colwell, 1974).

Of greater significance was the observed number of samples contaminated with *V. parahaemolyticus*. As indicated in Table 1, the lobster tail samples collected at the Fish Market in 1988 showed 62.5% of positivity to tests for microorganism. The positivity for those samples analyzed in 1990, although in smaller number is still high in comparison to the positivity of samples obtained from the processing plant (12.5%). Considering the samples from the Fish Market from both years (32), 19 (59.4%) of them were determined to be contaminated with *V. parahaemolyticus*.

Our results are similar to those of Hofer and Silva (1986), who detected the presence of *V. parahaemolyticus* in 45 (54.8%) out of 82 samples of various fish species, taken near shore off the Brazilian coast between Bahia and Rio Grande do Sul.

Lobsters despite being eaten cooked, may be a vehicle of *V. parahaemolyticus*, because the thermic treatment must be quick in order to preserve the texture of the muscle. It is important to the consumer to know that this crustacean must be eviscerated before cooking, because its bowel may contain a variety of microorganisms, including *V. parahaemolyticus*.

Nair et al. (1980) isolated this bacterium on 100% of fecal samples from marine fish.

Seng and Jegathesans (1977) isolated *V. parahaemolyticus* in cooked shrimp samples.

Although gastroenteritis reported to be caused by ingestion of food contaminated by *V. parahaemolyticus* has been associated mainly to oysters and crabs (Davis and Sizemore, 1982), Barker (1974) related a gastroenteritis outbreak in Massachusetts (USA), related to processed lobster, in which 31 out of 36 persons who ate the food became sick.

Table 2 records the K groups disclosed among *V. parahaemolyticus* strains isolated from lobster tail samples. Of 39 identified as *V. parahaemolyticus* in the samples from the Fish Market in 1988, 11 (28.2%) were "non agglutinable" and 28 (71.8%) were typable.

In addition, Table 2 shows that among the 14 isolates from the samples taken at Mucuripe in 1990, only 5 (35.7%) were typable and classified

TABLE 2 - Frequence of serogroups "K" among *Vibrio parahaemolyticus* strains isolated from lobster tails samples obtained in the permanent Fish Market at Mucuripe in 1988 and 1990 from a local fish industry, Fortalexa, Ceara, Brazil.

Serogroups	*V. parahaemolyticus* strains					
	Fish Market 1988		Fish Market 1990		Industry	
	Nº	%	Nº	%	Nº	%
KI	9	23.1	-	-	-	-
KII	4	10.2	-	-	-	-
KIII	1	2.6	2	14.3	1	33.3
KIV	5	12.8	2	14.3	-	-
KV	2	5.1	-	-	-	-
KVI	3	7.7	-	-	-	-
KVII	2	5.1	1	7.1	-	-
KVIII	1	2.6	-	-	-	-
KIX	1	2.6	-	-	-	-
NA*	11	28.2	9	64.3	2	66.7
Total	39	100.0	14	100.0	3	100.0

* "Non agglutinable" strains with polyvalent K-sera.

in the serogroups KIII, KIV and KVII, and the remaining 9 (64.3%) strains were "non agglutinable". The serogroup KIII was the only one present in both samples, from the Fish Market in 1988 and in those collected from the processor in 1990. Strains belonging to serogroups KIV and KVII were detected in samples from the Fish Market collected in 1988 and in 1990.

This multiplicity of serotypes in seafood was previously reported by Furniss et al. (1978). In addition, some authors (Hofer and Silva, 1986; De et al., 1977) have reported the occurrence of biochemically confirmed *V. parahaemolyticus* that did not agglutinate with the polyvalent antisera "K".

The results obtained in the Kanagawa test for all samples (Table 3) did not agree with those observed by some authors. For the samples collected at the Fish Market in 1988, of the 39 isolated strains of *V. parahaemolyticus*, 25 (64.1%) showed evidence of beta hemolysis production in the Wagatsuma culture medium. For those collected at the Fish Market in 1990, of the 14 isolated strains, 4 (28.5%) were Kanagawa-positive. No strain among 3 isolates from the processing industry samples showed positivity to this test. The same table shows that from 56 isolated strains of *V. parahaemolyticus*, 29 (51.8%) were Kanagawa-positive.

TABLE 3 - Distribution of the *Vibrio parahaemolyticus* strains according the Kanagawa test results and their respective percents. The lobster tails samples were obtained in the permanent Fish Market at Mucuripe in 1988 and 1990 and from a fish industry, Fortaleza, Ceara, Brazil.

Source	Total of isolates	*V. parahaemolyticus* Kanagawa-positive		Kanagawa-positive	
		Nº	%	Nº	%
Fish market 1988	39	25	64.1	14	35.9
Fish market 1990	14	4	28.5	10	71.4
Industry	3	-	-	3	100.0
Total	56	29	51.8	27	48.2

Binta et al. (1982) isolated *V. parahaemolyticus* on 53 out 584 samples of fish collected in the market at Quenia, and all the strains were Kanagawa-negative. Many other studies have reported that *Vibrio parahaemolyticus* strains isolated from sea foods or marine environments or both showed low results (of order 1%) or were negative for Kanagawa test (Sakazaki, 1968: Molenda, 1972).

However, Beartley and Slanetz (1971) isolated 50 strains of *V. parahaemolyticus* from oysters and water from seven different points of "Great Bay" and "Little Bay" in New Hampshire (USA) and found that all were positive to the Kanagawa test; and 15 of these strains, when tested for pathogenicity in mice, caused death of the animals within twelve hours.

Chun et al. (1974), in Korea, isolated 478 strains of *V. parahaemolyticus* from marine fish, and 39.7% produced the Kanagawa phenomenon in Wagatsuma agar.

Karanusagar and Mohankumar (1980) isolated 16 strains of *V. parahaemolyticus* from fishes, molluscs, estuarine water, and mangrove marsh, in India and found 4 (25%) to be Kanagawa-positive.

The statistical test showed no significant differences the positivities to *V. parahaemolyticus* between the samples collected at the Fish Market in 1988 and those collected there in 1990. The Kanagawa positivity for strains isolated from the samples taken in the Fish Market in 1988 (64.1%) was statistically different from that of their 1990 counterparts (28.6%) at the 5% level of significance.

The similarity in the contamination levels observed for the two years shows that the storage and sale conditions probably have not changed, and if so changes were incapable of diminishing the *V. parahaemolyticus* contamination of lobster tails.

The difference in the number of Kanagawa-positive strains found between the years 1988 and 1990 indicates only a quantitative difference in the capacity of the strains to cause the hemolysis of human erytrocytes, since both the Kanagawa-positive and Kanagawa-negative strains can be potentially pathogenic (Teramoto et al., 1971, Honda et al., 1987a, 1987b).

RESUMO

Detecção de *Vibrio parahaemolyticus* em cauda de lagosta, (*Panulirus laevicauda* L.).

Foi estudada a presença de *Vibrio parahaemolyticus* em 16 amostras de cauda de lagosta *Panulirus laevicauda* (Latreille), adquiridas na Feira de pescado do Mucuripe (Fortaleza, Ceará) em 1988, em 16 da mesma procedência em 1990 e em 16 provenientes de uma indústria de pescados local em 1988. O Número Mais Provável de *Vibrio parahaemolyticus* foi o teste utilizado para quantificação dessa bactéria. Os resultados variaram de 3,0 a 21,0/g. Das 39 cepas isoladas das amostras

da Feira em 1988, 11 (28,2%) mostraram-se "não aglutináveis" diante do soro anti-K utilizados para classificação em sorogrupos. Das cepas de *Vibrio parahaemolyticus* isoladas das amostras da Feira em 1990, somente 5 (35,7%) aglutinaram com o mesmo soro. Aquelas isoladas das amostras provenientes da indústria, somente uma (1) mostrou aglutinação. Os sorogrupos identificados entre as cepas foram: KI, KII, KIII, KIV, KV, KVI, KVII, KVIII e KIX. De um total de 56 cepas de *Vibrio parahaemolyticus* isoladas da feira em 1988 e 1990, 29 (51,8%) foram Kanagawa-positivas enquanto que as 3 isoladas da amostra da indústria foram negativas. Entre as cepas Kanagawa-positivas 25 (64%) foram isoladas da Feira de Pescado em 1988 e 4 (28,6%) em 1990.

Palavras-Chave:*Vibrio parahaemolyticus:* lagosta, teste Kanagawa.

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SALMONELLA IN POULTRY FEEDS IN BRAZIL

A. Berchieri Jr.[1]*
S. A. Fernandes[2]
K. Irino[2]
J. L. Quintana[1]
A. J. Santos[1]

SUMMARY

This experiment was undertaken to determine the possible presence of *Salmonella* in poultry diets. A total of two hundred samples of ration from 4 commercial poultry feed industries were examined. The results revealed the presence of salmonellae in 10% of the samples studied and 14 serotypes were identified. The procedure for *Salmonella* isolation included the pre-enrichment step and the strains were submitted to antimicrobial tests. The 29 strains were resistant to the followings antimicrobial agents (% of resistance in parenthesis): Erythromycin (100%), sulphonamides (100%), colistin (100%), streptomycin (100%), bacitracin (100%), penicillin (100%), tetracycline (92,9%), cephalothin (75%), carbenicillin (62,5%), ampicillin (46,5%), kanamycin (46,5%), nitrofurantoin (39,3%), neomycin (25%), amikacin (21,4%), sulphazotrin (21,4%), nalidix acid (18,8%), chloramphenycol (17,9%), linco-spectin (17,9%), gentamicin (17,9%), and cefoxitin (6,3%).

Key words: *Salmonella,* isolation, poultry feeds, antimicrobials.

INTRODUCTION

Salmonella organisms continue to be a very serious cause of human food-poisoning. Poultry meat is often considered to be a source of food-borne *Salmonella* infection (9) which chickens acquire through contaminated feed (15). Poultry diet becomes contaminated mainly by infected feedstuff of animal origin (14). In Brazil, there is little information available about the damage that *Salmonella* can cause to public and animal health. However, the presence of *Salmonella* in animal poultry feed ingredients, in poultry abbatoirs and in human beings has been reported by several investigators (2, 3, 6, 10).

Since previous works have shown that feedstuffs of animal origin are contaminated with salmonellae, determining the sources of *Salmonella* infection in chickens such as the ration would be helpful in preventing infection of animals and food-poisoning. Thus, the present experiment was undertaken to study the presence of salmonellae in poultry diets of commercial origin.

MATERIALS AND METHODS

Salmonella was isolated by the method used by Berchieri Jr. et al. (4). A total of 200 samples of

1. Faculdade de Ciências Agrárias e Veterinárias campus de Jaboticabal - UNESP (FCAVJ-UNESP), Rodovia Carlos Tonanni - Km 5 - 14870-000 Jaboticabal SP Brazil. Deptº de Patologia Veterinária (Ornitopatologia).
2. Instituto Adolfo Lutz, São Paulo-SP, Brazil.
* Corresponding Author

mashed feeds from 4 commercial poultry feed industries were examined. Four 25 gm amounts of each sample were examined as follows: 2 were added into 2 x 225 ml of Ringer's solution 1/4 (RS 1/4) and 2 were added into 2 x 225 ml of lactose broth (LB), and all of them were kept at room temperature for 6 hours. One bottle containing RS 1/4 and one containing LB were then incubated overnight at 37°C and at 43°C respectively. Aliquots (2 x 2 ml) from each bottle were added into 20 ml of selenite-novobiocin (40 mg/l) broth or into 20 ml of tetrathionate-novobiocin (40 mg/l) broth. The enrichment broths were incubated for 24 hours at the same temperature used for the previous pre-enrichment. The enrichment broths were plated on brilliant green agar and on MacConkey agar which were incubated overnight at 37°C. *Salmonella*-like colonies were inoculated into IAL medium and incubated overnight at 37°C. The serotypes were identified at Instituto Adolfo Lutz, São Paulo, Brazil.

The sensitivity of the 29 isolated strains to the antimicrobial agents was tested by the method of Bauer et al. (1), using discs (CEFAR) containing the following drugs: amikacin (30 mcg), ampicillin (10 mcg), bacitracin (10 UN), carbenicillin (100 mcg), cephalothin (30 mcg), cefoxitin (30 mcg), chloramphenycol (30 mcg), colistin (10 mcg), erythromycin (15 mcg), gentamicin (10 mcg), kanamycin (30 mcg), linco-spectin (2/30 mcg), nalidixic acid (30 mcg), neomycin (30 mcg), nitrofurantoin (300 mcg), penicillin (100 UN), streptomycin (10 mcg), sulphazotrin (25 mcg), sulphonamides (300 mcg) and tetracycline (30 mcg).

RESULTS

Table 1 shows the distribution of *Salmonella* contamination by commercial origin of the diets and Table 2 shows the serotypes isolated from the contaminated samples. Twenty-nine strains of 14 serotypes were detected. The results obtained using two different pre-enrichment procedures and temperatures are presented in Table 3. The antibiogram of the 29 strains showed the following pattern of resistance: 100% to erythromycin, sulphonamides, colistin, streptomycin, bacitracin and penicillin, 92.86% to tetracycline, 75% to cephalo-

TABLE 1 - Isolation of *Salmonella* serotypes from diets obtained from 4 feeds mills.

Feed mill	Number of samples		
	Tested	Positive for *Salmonella*	%
A	50	13	26
B	50	5	10
C	50	2	4
D	50	0	0
Total	200	20	10

TABLE 3 - Isolation of *Salmonella* considering the pre-enrichment and the temperature.

Method	Number of samples	
	Positive	Percentage (%)
RS 1/4[1] 37°C	5	2,1
LB[2] 37°C	3	1,5
RS 1/4 43°C	14	7,0
LB 43°C	12	6,0

1 Ringer's solution 1/4
2 Lactose broth

TABLE 2 - *Salmonella* serotypes isolated from each positive sample.

Sample	Mill	*Salmonella*	Sample	Mill	*Salmonella*
01	B[1]	*S. mbandaka*	04	A	*S. cerro*
04	A	*S. adelaide*	93	A	*S.* I 6,7:r:-
08	B	*S.anatum, S. mbandaka*	123	A	*S. mbandaka*
16	B	*S. anatum, S. mbandaka*	129	A	*S. anatum, S. mbandaka*
17	C	*S.* I 3, 15:y:	130	A	*S.cerro, S. montevideo*
19	A	*S. adelaide*	131	B	*S. anatum* LDC-2
20	A	*S. adelaide, S. meleagridis* and *S. minnesota*	133	A	*S.* I 3,10:-:1,6 LDC- *S.* I 4,12:d:- *S. anatum* LDC-
32	A	*S. cerro* and *S. mbandaka*	136	C	*S. anatum* LDC-
33	A	*S. cerro*	139	A	*S. anatum* LDC-
51	B	*S. mbandaka*	147	A	*S. lille*

1 For sample origin see Table 1
2 Lysine descarboxylase negative

thin, 62.5% to carbenicillin, 46.5% to ampicillin and kanamycin, 39.3% to nitrofurantoin, 25% to neomycin, 21.4% to amikacin and sulphazotrin, 18.8% to nalidixic acid, 17.9% to chloramphenycol and linco-spectin, 17.9% to gentamicin and 6.3% to cefoxitin.

DISCUSSION

Feeds used at poultry farms are often infected with *Salmonella* (14). The present study revealed that 10% of the 200 diet samples studied were contaminated with one, two or three *Salmonella* serotypes, with different degrees of contamination among feeds from different industries. The isolation of salmonellae from poultry feed was not surprising since previous studies have shown the presence of the bacteria in by-products of animal origin used to prepare feeds (2, 3, 4, 8, 11). Among the strains isolated multiple resistance was a frequent possibly due to the lack of control on the addition of either feed additives or growth promoters. Such promoters have been recognized as a factor leading to prolonged faecal excretion of *Salmonella* (12) and also, they can provoke the emergence of multiple drug-resistance, which could be associated with the enhancing of virulence to the host by the bacteria (13). The approach used here to isolate salmonellae using inicially a pre-enrichment step showed that the best results were obtained when the samples were added into the RS 1/4 and incubated at 43°C. However, this method was not able to isolate the bacteria from all positive feed samples, suggesting that negative results do not mean absence of salmonellae organisms in the feed.

Since the animal by-products used to prepare ration are a very common source of Salmonella, presumably the best way to detect *Salmonella* would be the microbiological investigation toward to feed ingredients.

The results of this experiment showed the presence of *Salmonella* in the poultry feeds. Since the detection is dependent on the presence of the bacteria in the diet samples, negative results do not mean the absence of bacteria in the diet. Thus, it is suggested to analyse preferentially the feeds ingredients of animal origin before reaching the feed industry. At the present moment a good program to control *Salmonella* should contain general measures of prophylaxis to attack all links of the epidemiologic chain of salmonellosis in which must be included a educational program similar that suggested by Brown (7).

RESUMO

Salmonella em Ração para Aves no Brasil

Foram examinadas 200 amostras de ração de quatro diferentes empresas. Vinte amostras estavam contaminadas por *Salmonella*. As cepas isoladas mostraram os seguintes percentuais de resistência a antimicrobianos: 100% (eritromicina, sulfonamida, colistina, estreptomicina, bacitracina e penicilina), 92,86% (tetraciclina), 75% (cefalotina), 62,5% (carbenicilina), 46,5% (ampicilina e canamicina), 39,39% (nitrofurantoína), 25% (neomicina), 21,4% (amicacina e sulfazotrim), 18,8% (ácido nalidíxico), 17,9% (cloranfenicol, lincospectin, gentamicina) e 6,3% (cefoxitina).

Palavras-chave: Salmonella, isolamento, ração para aves, antimicrobiano.

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USE OF *BACILLUS SUBTILIS* SPORES TO EVALUATE THE EFFICIENCY OF SODIUM HYPOCHLORITE AT DIFFERENT CONCENTRATION AND PH VALUES

Nélio José de Andrade[1]
António de Melo Serrano[2]

SUMMARY

The sporicidal action of NaOCl at 50, 75, 105, 150 and 200 mg/l of free available chlorine (FAC) at pH 9.8 and 105 mg/l at pH 7.0, 8.0 and 9.0, was studied using lots of spores of *Bacillus subtilis* ATCC 19659 suspended in water with 15% v/v glicerol at 30°C. The contact times were chosen from a death range in preliminary studies. The decimal reduction (DR) in the number of spores subjected to contact with the hypochlorite solutions was determined by difference between the decimal logarithm (log) of the initial number spores (not submitted to the solution) and the surviving spores after each time contact. Simple linear regression models were fitted to the DR values as function of spore contact time in each solution. These equations were used to estimate the spore initial death time (SIDT) and D value of each solution. A linear relation was found between the log of SIDT (Y1), in minutes, the log of D values (Y2), in minutes and the log of hypochlorous acid concentration (G), in mg/l. The equations G= mg/l FAC/1 + $10^{pH - 7,50,}$ Y1 = -1.0945G + 1.5106 (r^2 = 0.99) and Y2 = -0.8869G + 1.1401 (r^2 = 0.99) were rearranged and transformed by software "DERIVE" for algebric simplification originating a fourth equation to estimate the NaOCl solution contact time (T) required to produce a given DR at a given concentration of FAC an pH value:
$T = 32.4026\ e^{-2.52014G} + 13.8065\ DR\ e^{-2.04213G,}$
where e = neperian base; 50mg/l <FAC<200mg/l, at 30°C; 7.0<pH<9.8

Key words: Sanitizer efficiency; Sodium Hypochlorite; Sporicidal action; *Bacillus subtilis* spores; Biological indicator.

INTRODUCTION

There is a number of microbiological test for evaluating sanitizing agents. Among them, the Suspension Test, also referred to as Chambers Test and Sanitizer Efficiency Test, is widely recommended in the food industries (4,7,8,9,10,12). It is a simple test permitting simulation of the plant conditions (10) and domestic use (18).

The Suspension Test does not differentiate between the efficiency of chlorinated solutions recommended for sanitizing walls and floors of processing plant, processing equipments and utensils. Free available chlorine (FAC) concentration in the range of 50 to 200 mg/l at pH between 7.0 and 11.0, from fast-acting compounds as sodium hypochlorite, easily reduces the number of *Staphylococcus aureus* ATCC 6538 and *Escherichia coli*

1. Food Technology Department/UFV - 36570 - Viçosa - MG.
2. Food Technology Department/UNICAMP - 13081 - Campinas - SP.

ATCC 11229, within 30 seconds of contact at 20°C, as recomended by the Suspension Test (12).

Due to lack of differential sensitivity at high hypochlorite concentration, *Bacillus subtilis* ATCC 19659 spores were used in this study as substitute for vegetative cell, in evaluating sanitizing efficiency. These spores are more resistant to hypochlorite than other food pathogenic microorganisms such as *Clostridium botulinum, Clostridium perfringens* and *Bacillus cereus* (1,2,3,5,12,15,16). Another goal was to develop an equation to estimate contact time at given sanitizing condition.

MATERIALS AND METHODS

Lots L, M, N, O, P and Q of *B. subtilis* ATCC 19659 spores were produced according to procedure recommended by the National Food Processors Association (14), with modifications. The spores were developed on sporulation medium surfaces [3.0g beef extract (Difco), 1.0 g tryptone (Difco), 1.0 g Starch (Reagen), 10.0 mg manganese sulphate (Carlo Erba) and 20.0 g agar (Difco) in 1000 ml of distilled water]. The reaction was adjusted to 7.0±0.1 and sterilized at 121°C/15 min. The incubation was at 30°C/96h, at the end of this time more than 90% sporulation was achieved.

The spores on the medium surface were suspended in sterilized distilled water and centrifuged at 2500Xg/15 min at 4°C. This step was repeated 5 times for each lot of spores resulting in a concentration of approximately 10^9 spores/ml, which was finally suspended in water with 15% v/v glicerol and stored at 4°C for use in subsequent experiments.

The spore lots L, M and N were exposed to solutions of sodium hypochlorite prepared with 0.067M phosphate buffer pH 9.8 at 30°C and concentrations of 50, 75, 105, 150 and 200 mg/l of FAC, as measured by iodometric method (17).

The remaining spore lots (O, P e Q) were also prepared in the same way with the only difference that they were exposed to solutions with 105 mg/l of FAC at pH values of 9.0, 8.0 and 7.0

The sporicide action of each of these solutions was evaluated as follows: to 99 ml of each solution was added 1.0 ml of a heat-treated (85°C/15 min in order to eliminate the vegetative cell) portion of the spore suspension. At pH 9.8, the contact time for 50 mg/l of FAC was fixed at 140, 180, 220, 240, 260 and 290 min. In another series of treatments, the previous pH value was maintained, but the hypochlorite solutions concentration increased to 75 mg/l besides changing the contact time to 80, 110, 140, 170, 190 and 220 min; at 105 mg/l, the contact times were 60, 90, 120, 140, 160 and 180 min; at 150 mg/l, the contact times were 50, 65, 80, 95, 110 and 120 min; and finally 40, 50, 60, 70, and 80 min, for 200 mg/l of FAC. In the case of 105 mg/l of FAC the contact times were fixed at 10, 15, 20, 25, 30, and 35 min for pH 9.0; 1, 2, 3, 4 and 5 min for pH 8.0 and 0.33, 0.66, 0.83, 1.00, 1.13, and 1.33 min for pH 7.0. After these contact times-established through previous trials of logarithmic death range for each solution - 1.0 ml of hypochlorite solutions with the spores was transfered to test tube containing 9.0 ml of the neutralizing sodium thiosulphate solutions 0.25% w/v in 1:4 Ringer solution. Dilutions of this neutralized spore suspension were immediately plated out in duplicate in plate count agar (PCA). The colonies were counted after 48h incubation at 30°C.

The decimal reduction (DR) in the number of spores subjected to contact with the hypochlorite solutions was determined by difference between the decimal logarithm of the initial number of spores (not submitted to the solution) and the surviving spores after each contact time. Simple linear regression models were fitted to the DR values as function of spore contact time in each solution. These equations were used to estimate the spore initial death time (SIDT) of each solution, attributing the value zero (0) for Y. The D values were estimated from the difference between the time necessary to obtain the consecutive D value or by the inverse of the straight line slope determined by the regression equation.

RESULTS AND DISCUSSION

From the experimental DR values shown in Table 1, it was possible to determine the linear regression equation $Y = aX + b$, where Y represents the DR values and X the contact time of hypochlorite solution with the spore suspension (Table 2). From the linear regression the SIDT and D values were estimated (Table 3).

It can be observed from Table 3 that increasing in the FAC concentration in the solution or lowering its pH value decreases the SIDT and D values. Besides concentration and pH, other factors such as instability of chlorine under storage (7), type and number of microorganism, organic matter, time and temperature of contact (5,12,15) influences the sporicide action of the chlorinated solutions. In the present work these action was dependent on the concentration and pH of the chlorinated solutions since those other factors were kept constant.

TABLE 1 - Decimal reduction numbers of *Bacillus subtilis* spores after contact times (underlined) exposed to sodium hypochlorite solutions (NaOCl) at several pH values and 30°C. Mean values of three replicates for each lot.

NaOCl (mg/l)	pH	*B. subtilis* Spores Lots	Contact time (min)					
			140	180	220	240	260	290
50	9.8	L	0.21	0.64	2.28	–	2.70	3.26
		M	0.19	0.60	1.59	2.69	–	3.34
		N	0.26	0.60	2.29	–	2.46	3.23
			80	110	140	170	190	220
75	9.8	L	0.14	0.27	0.75	1.98	2.85	3.34
		M	0.13	0.31	0.73	2.10	3.21	3.84
		N	0.10	0.35	0.77	2.22	3.01	3.58
			60	90	120	140	160	180
105	9.8	L	0.18	0.84	2.15	3.00	3.55	4.34
		M	0.22	0.76	2.23	2.95	3.45	4.22
		N	0.18	0.80	2.20	2.90	3.35	4.31
			50	65	80	95	110	120
150	9.8	L	0.15	0.93	2.42	3.37	3.99	–
		M	0.13	0.58	1.71	2.81	3.50	4.17
		N	0.15	0.60	1.69	2.80	–	4.15
			40	50	55	60	70	80
200	9.8	L	0.38	1.20	–	2.64	3.45	4.29
		M	0.36	1.23	2.06	2.48	3.53	–
		N	0.42	1.32	2.23	2.68	–	–
			10	15	20	25	30	35
105	9.0	O	0.10	0.36	1.34	2.52	3.38	4.00
		P	0.13	0.39	2.05	2.88	3.48	4.14
		Q	0.17	0.33	1.77	2.52	3.60	4.28
			1	2	3	4	5	
105	8.0	O	0.25	0.87	2.48	3.39	3.75	
		P	0.09	0.76	2.41	3.42	4.12	
		Q	0.23	1.29	3.51	4.09	4.67	
			0.33	0.66	0.83	1.00	1.13	1.33
105	7.0	O	0.21	1.22	2.38	2.98	3.83	4.24
		P	0.28	1.21	2.42	2.83	3.45	4.04
		Q	0.20	1.23	2.48	2.69	–	3.47

- Not determined.

It is known that sodium hypochlorite as well as other chlorinated compounds with the exception of chlorine dioxide (6, 15) liberate hypochlorous acid in aqueous solutions, which represents pronounced sporicide effect (5,12,15,16,19,20). The hypochlorous acid concentration can be calculated from the rearrangement of the Henderson-Hasselbalch equation (1) which interrelates the constant of dissociation of hypochlorous acid [Ka = 3.18×10^{-8} at 30°C (13); pKa = 7.50] and the concentration of FAC and solution pH value:

$$\text{Mg/l HOCL} = \frac{\text{mg/l FAC}}{1 + 10^{\text{pH} - 7.50}} \qquad \text{(Equation 1)}$$

TABLE 2 - Linear Regression of decimal reduction number of *Bacillus subtilis* spores exposed to chlorinated solution at several concentrations and pH values at 30°C.

NaOCl (mg/l)	pH	Linear Regression[a]	R^2
50	9.8	Y = 0.0216X - 2.915[b]	0.94
75	9.8	Y = 0.0272X - 2.472	0.92
105	9.8	Y = 0.0351X - 2.073	0.95
150	9.8	Y = 0.0611X - 3.011	0.95
200	9.8	Y = 0.1036X - 3.731	0.96
105	9.0	Y = 0.1733X - 1.819	0.97
105	8.0	Y = 1.0640X - 0.836	0.91
105	7.0	Y = 0.0648X - 1.077	0.95

a - Coefficient X significant at 5% probability level ($p<0.05$).
b - Y = Decimal Reductions.
X = Contact Time in minutes.

TABLE 3 - Initial death time and D values for the *Bacillus subtilis* spores exposed to several hypoclorus acid concentrations (HOCl).

NaOCl (mg/l)	pH	HOCl* (mg/l)	Initial Death** Time (min)	Value D** (min)
50	9.8	0.25	134.95	46.30
75	9.8	0.37	91.03	36.76
105	9.8	0.52	59.06	28.49
150	9.8	0.75	49.28	16.37
200	9.8	1.00	36.01	9.66
105	9.0	3.22	10.50	5.77
105	8.0	25.24	0.79	0.94
105	7.0	79.55	0.28	0.25

* - by Henderson-Hasselbalch Equation.
** - by linear regressions Equations.

It is important to note that the information on the quantity of FAC solution, usually determined by conventional titrimetric method is not enough to predict its sporicide efficiency. The expression FAC means the concentration of hypochlorous acid plus hypochlorite ions in solution. The concentration of each component can change as function of pH. Thus, FAC and pH values are mutually dependent and both determine the availability of hypochlorous acid for spore destruction.

From the previous equation, it can be concluded that solutions with pH values equal to pKa (7.50) have 50% of FAC as hypochlorous acid while in solutions of pH values below the pKa the hypochlorous acid predominant; the reverse is true concerning solutions with pH above the pKa in which the hypochlorite ion is in much higher concentration.

Equation 1 explains the effects of observed concentration and pH. When the concentration was increased from 50 to 200 mg/l of FAC at constant pH there was a corresponding increase in the level of hypochlorous acid, as the equation numerator also increased. The pH values decrease to 9.0, 8.0 and 7.0 resulted in smaller denominator and consequently the solutions also showed higher contents of hypochlorous acid, thus more sporicide efficiency.

It can be easily noted from Table 3 that increases in the hypochlorous acid concentration resulted in reduction in the SIDT and D values. Based on the literature (15, 16) these results were analysed and simple linear regressions between the decimal logarithm of SIDT, the decimal logarithm of D values and decimal logarithm of hypochlorous acid concentration were fitted (Figure 1). The linear regression, Equation 2 , was determined as

$$Y_1 = -1.0945G + 1.5106 \qquad \text{(Equation 2)}$$

where Y_1 is the SIDT decimal logarithm, in minutes, for the *B. subtilis* spores; G is the decimal logarithm of concentration of hypochlorous acid in mg/l (Figure 1).

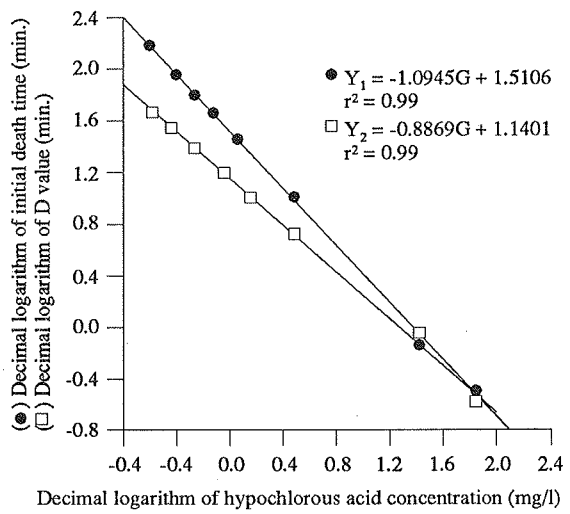

FIGURE 1 - Linear regression of decimal logarithm of initial death time and decimal logarithm of D value of *Bacillus subtilis* spores in function of decimal logarithm of hypochlorous acid concentration

The coefficient of determination was 0.99 meaning that 99 % the variation in the SIDT decimal logarithm could be explained by variation in hypochlorous acid concentration decimal logarithm.

Similarly, the linear regression, Equation 3,

$$Y_2 = -0.8869G + 1.1401$$

relates decimal logarithm value of D (Y), in minutes, of the *B. subtilis* spores with the decimal logarithmic value of the concentration of hypochlorous acid (G), in mg/l, (Figure 1). For this Equation the coefficient of determination was also 0.99.

Finally, the Equations 1, 2 and 3 were rearranged and transformed by the software "DERIVE" for algebric simplification originating a fourth Equation:

$$T = 32.4026\, e^{-2.52014G} + 13.8065\, DR\, e^{-2.04213G},$$

(Equation 4)

where:

T = contact time of the sanitizing solution with the spores;

DR = required decimal reduction values in number of spores;

e = neperian logarithm base

$$G = \log\left(\frac{\text{mg/l of FAC}}{1 + 10^{pH - 7.50}}\right)$$

where $50 < \text{mg/l FAC} < 200$, at 30°C

$7.0 < pH < 9.8$

If "DR" is taken as zero, it is possible to obtain the time for the initial spore death within limits of experimental conditions. On the other hand, considering the second term of the Equation, it is possible to obtain the D value.

The contact time obtained through Equation 4 is valid only when the sporicide action is basically dependent on FAC concentration and pH value of the solution as occurred in this experiment. It is therefore valid to use this procedure in evaluating the sporicide power of sodium hypochlorite.

This equation is to apply in situations occurring between the logarithm destruction range. Nevertheless the chlorine-treated surviving spores curve may present a "shoulder" (lag phase) and/or a "tail" (6, 11). The lag phase occurs at the beginning of the treatment, particularly when low hypochlorous acid concentrations are observed and is due to the chemically resistant spore coat. The "tail" appears at the end of the treatment and might be explained through spore clumping and heterogeneity, as well as changing resistance during the chemical attack, among others.

However, even in industrial conditions, where other factors may influence the sporicide action of chlorinated compounds, there is no doubt that the values estimated through by Equation 4, may can be used as a criterium for fixing the contact time required for eliminating undesired spores and vegetative cells in routine sanitary procedures.

RESUMO

Uso de esporos de *Bacillus subtilis* para avaliar a eficiência do hipoclorito de sódio em diferentes concentrações e valores de pH.

A ação esporocida de soluções de hipoclorito de sódio (NaOCl) contendo 50, 75, 105, 150, e 200 mg/l de cloro residual livre (CRL) em pH 9,8 e 105 mg/l em pH 9,0, 8,0 e 7,0 à temperatura de 30°C foi avaliada em lotes de esporos de *Bacillus subtilis* ATCC 19659 suspensos em glicerol e água (15:85). Os tempos de contato, para cada condição, foram escolhidos dentro da faixa de morte logarítimica, encontrados em ensaios preliminares. As reduções decimais (RD) no número de esporos submetidos ao contato com as soluções de NaOCl foram determinadas pela diferença entre o logarítmo decimal (log) do número inicial (esporos não submetidos às soluções) e o número de sobreviventes após cada tempo de contato. Equações de regressão linear foram ajustadas para cálculo das RD em função do tempo de contato para cada situação. A partir destas equações, foram determinados o tempo de início de morte (TIM) e o valor D para cada solução. Obteve-se também uma relação linear entre o log do TIM (Y1), em minutos, o log dos valores D (Y2), em minutos e o log da concentração de ácido hipocloroso (G), em mg/l. As equações $G = \text{mg/l CRL/1} + 10^{pH - 7.50}$, $Y1 = -1,0945 + 1,5106$ (r^2=0,99) e $Y2 = -0,8869G + 1,1401$ ($r^2 = 0,99$) foram rearranjadas e transformadas pelo "software DERIVE" para uma simplificação algébrica, originando uma quarta equação, que estima o tempo de contato do NaOCl para se obter uma determinada RD no número de esporos a uma dada concentração de CRL e o valor de pH das soluções:

$T = 32,4026\, e^{-2,52014G} + 13,8065\, RD\, e^{-2,04213G}$,

em que e = base do logarítmo neperiano; 50mg/l<CRL<200mg/l, a 30°C; 7,0<pH<9,8.

Palavras-chave: Eficiência de sanificantes; Hipoclorito de sódio; Ação esporicida; *Bacillus subtilis;* Indicador biológico.

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INFLUENCE OF TEMPERATURE AND RELATIVE HUMIDITY IN THE PRODUCTION OF AFLATOXINS IN SAMPLES OF STORED MAIZE, ARTIFICIALLY CONTAMINED WITH *ASPERGILLUS FLAVUS* (LINK).

Asevedo, I. G.
Gambale, W.
Corrêa, B.
Paula, C. R.
Almeida, R. M. A.
Framil, V. M. S.

SUMMARY

Ninety (90) samples of maize grains from several regions of Brazil were utilized in order to verify the influence of temperature and relative humidity in the production of aflatoxins in this substract. The samples were inoculated with toxigenic *A. flavus* and incubated in a controlled temperature and relative humidity environment (15°C and 61% RH, 86.5% RH, 99% RH; 25°C and 64% RH, 85% RH; 40°C and 61.5% RH, 85.5% RH; 96% RH) for period of 10, 20 and 30 days of incubation. Aflatoxins were detected in the incubated samples at 15°C and 86.5% RH; 25°C in the three humidity conditions studied, 40°C and 61.5% RH, 96% RH. The best conditions for the production of aflatoxins were 25°C and 85% RH and 98% RH. The lowest levels were obtained at 40°C and 61.5% RH. It was thus concluded that the lowest temperatures (15°C) with. A relative humidity of 61% and moisture content of 13.0% seem to be best conditions for the storage of maize.

Key words: *Zea mays* (L.). stored maize, *Aspergillus flavus*, aflatoxins, temperature, relative humidity, moisture.

INTRODUCTION

Maize (corn) is one of the most spreaded crops in Brazil, with nearly 13 million hectares planted (IBGE). Most part is destined to feed animals as such or as part of a ration, in which it can be up to 63% of the formulation for poultry and 75% for hogs (9).

Maize, such as other products from vegetal origin, is subject to the natural contamination of vegetal and soil fungi. Several of these fungi are potential producers of mycotoxins (19).

Like other cereals, maize can be stored for long periods of time after it is dry. However if the conditions of storage are not adequate, those fungi from natural origin as well as others acquired during the manipulation of products can grow and produce mycotoxins (19).

After peanut, maize is the product which is mostly mentioned in world literature regarding its

Institute of Biomedical Sciences
University of São Paulo, Brazil - Department of Microbiology.
05508 - 900 - São Paulo, Brazil.

contamination by aflatoxins (19). In Brazil, in spite of the great consumption and the adequate climatic conditions for the development of fungi producers of aflatoxins, the existing publications refer only to the detection of mycotoxins in maize and by products (10, 11, 12, 16, 17).

Considering this, the objetcive of the present study was to verify the influence of temperature, and relative humidity in the production of aflatoxins by *Aspergillus flavus* in maize, from storage silos, under laboratorial conditions.

MATERIAL AND METHODS

Samples - 90 samples of maize grain, from various regions of Brazil with aflatoxins not detectable, were utilized.

Culture of toxigenic A. flavus - The inoculation of maize grains was performed with a culture of A. *flavus*, producer of aflatoxins B1 and G1.

Suspension of spores - The suspension of spores used for the inoculation of corn grain samples was obtained from 7 sterilized day cultures of *A. flavus* in Czapeck agar at 25°C, by the addition of sterilized distilled water. The spores were removed with a platinum handle and the number of spores in the suspension was 1 x 10^7 spores/ml, as per counting in the Neubauer chamber.

Determination of the moisture content (MC) - The moisture content (MC) of maize grains was determined before and after incubation, through a Elotest apparatus, model 777.

Incubation of the samples - 350 g of maize samples were placed in glass flasks (18 cm of diameter per 8 cm of height) and inoculated with 1.10^7 spores/ml of toxigenic *A. flavus* placed in incubating ovens model BOD, inside poliethilene containers sealed with transparent PVC film in order to maintain the relative humidity according to Table 1 (18). The time of incubation established was a minimum period of 10 days, a medium period of 20 days and the maximum period of 30 days.

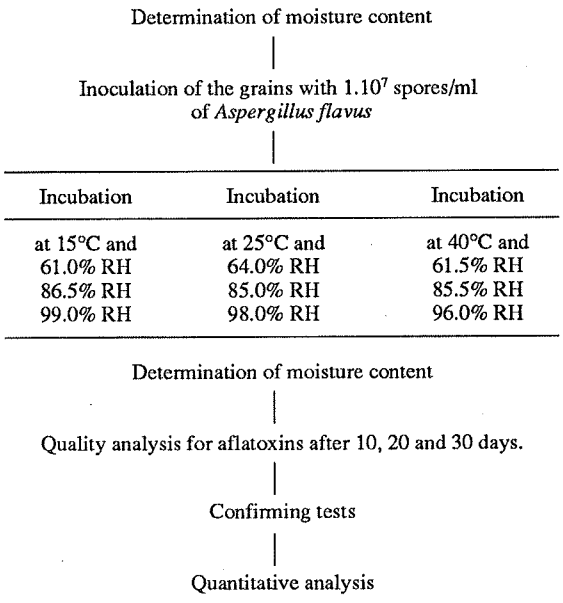

FIGURE 1- Schematic representation of the procedure.

TABLE 1 - Relative himidity of the environment obtained with higrostatic salts in relation to temperature of 15,25 and 40°C.

T (°)	RH (%)	Higrostatic Salts
15	61.0	$NaBr_2H_2O$
	86.5	KCL
	99.0	K_2SO_4
25	64.0	$NaNO_2$
	85.0	KCl
	98.0	$K_2Cr_2O_7$
40	61.5	$NaNO_2$
	85.5	K_2CrO_7
	96.0	$K_2Cr_2O_7$

T = Temperature
RH = Relative humidity

Determination of aflatoxins - Toxins extraction, cleanup, thin-layer chromatography were based in the analytical norms of Instituto Adolfo Lutz (13). Quantitative analysis was made using dilution to extintion technique (3).

TLC Confirmation of aflatoxins - With the positive qualitative analysis, the following confirmatory tests were performed: development of the ethyl ether in the chromatographic plate; spraying sulfuric acid solution at 50% on the spots to observe the change in the blue fluorescence (aflatoxin B1) to yellow, and two-dimensional chromatography.

RESULTS AND DISCUSSION

The results are presented on Tables 2, 3 and 4.

At 15°C there was production of aflatoxins only in the samples incubated at 86.5% of relative humidity and MC between 17.0% and 18.8% (pre-incubation) and between 19.4% and 25% (post-incubation of 10 days), values considered optimum for the production of aflatoxins in cereal

TABLE 2 - Moisture of the grain and production of aflatoxins in 30 samples of maize from storage inoculated with toxigenic *Aspergillus flavus* at 15°C and 61, 86.5 and 99% of relative humidity after 10, 20, and 30 days of incubation.

	Samples	Moisture pre incubation (%)	Moisture pos incubation (%)	Qualitative Analysis for aflatoxins 10	20	30 (Days)	Level of aflatoxins B1	G1 (ppm)	Total of B1 + G1 (ppm)
	1	13.0	13.0	-	-	-	-	-	-
	2	12.0	12.0	-	-	-	-	-	-
	3	12.0	11.9	-	-	-	-	-	-
	4	12.0	12.0	-	-	-	-	-	-
61%	5	12.0	12.0	-	-	-	-	-	-
Humidity	6	12.2	12.0	-	-	-	-	-	-
	7	12.1	12.0	-	-	-	-	-	-
	8	12.3	11.9	-	-	-	-	-	-
	9	12.0	12.0	-	-	-	-	-	-
	10	12.0	11.0	-	-	-	-	-	-
	11	18.5	19.4	+			0.50	0.02	0.52
	12	17.5	25.0	+			0.13	0.04	0.17
	13	17.8	19.9	+			0.20	0.04	0.24
	14	17.2	19.8	+			0.06		0.06
86.5%	15	18.8	20.7	+			0.60	0.12	0.72
Humidity	16	17.8	23.8	+			0.70	0.12	0.82
	17	18.0	23.0	+			0.70	0.12	0.82
	18	17.0	21.6	+			0.20	0.08	0.28
	19	18.1	21.2	+			0.16	0.08	0.24
	20	18.7	20.6	+					
	21	12.0	16.3	-	-	-	-	-	-
	22	12.3	16.3	-	-	-	-	-	-
	23	12.0	17.0	-	-	-	-	-	-
	24	12.0	15.9	-	-	-	-	-	-
99%	25	12.2	15.8	-	-	-	-	-	-
Humidity	26	12.1	18.3	-	-	-	-	-	-
	27	12.3	18.3	-	-	-	-	-	-
	28	12.0	17.8	-	-	-	-	-	-
	29	12.0	17.0	-	-	-	-	-	-
	30	12.1	17.9	-	-	-	-	-	-

(+) = Detectable
(-) = Not detectable
Obs.: The samples which were positive in 10 days were not analyzed after 20 and 30 days.

grains (1). It is possible that these high levels of MC must have been a fundamental factor for the production of aflatoxins, since the environment of 61% and 99% of relative humidity with the same temperature (15°C) there was no production of aflatoxins. In both situations, the moisture of the samples, pre-incubation, was between 12 and 13%, levels recommended by the Food Agriculture Organization 1979 to avoid the production of aflatoxins.

At 25°C and 64% RH there was production of aflatoxins in 50% of the samples after 10 days. Is noted that these samples, with humidity relatively high, before incubation, had these values diminished after 10 days, but continued over 13.5%. After 30 days of incubation, more than 40% of the samples were positive and the MC of these samples did not surpass 13.5%, which was in disagreement with the recommendation of the Food and Agriculture Organization, that maize is protected from the production of aflatoxins when the grains are stored at 27°C, MC of 13.5% and RH of 70%.

At 25°C and 85% and 98% RH, it was observed a production of aflatoxins after 10 days of incubation in all the samples studied. This observation agrees with the findings of other authors (2, 4, 5, 6, 14, 15) that 25°C - 27°C is the best temperature for the production of aflatoxins.

At 40°C and 61.5% RH there was a decline in the levels of MC between 12% and 14.7%, considered unfavorable to the production of aflatoxins in

TABLE 3 - Moisture content of the grain and production of aflatoxin in 30 samples of maize from storage, inoculated with toxigenic *Aspergillus flavus* at 25°C and 64, 85 and 98% of relative humidity after 10, 20 and 30 days of incubation.

	Samples	Moisture pre incubation (%)	Moisture pos incubation (%)	Qualitative Analysis for aflatoxins 10	20 (days)	30	Level of aflatoxins B1 (ppm)	G1	Total of B1 + G1 (ppm)
	31	15.0	12.8	-	-	+	0.10	0.20	0.30
	32	14.9	13.0	-	-	+	0.10	0.20	0.30
	33	16.2	14.7	+			0.10	0.20	0.30
	34	16.3	16.0	+			0.10	0.20	0.30
64%	35	15.9	15.4	+			0.20	0.20	0.40
Humidity	36	16.0	13.2	-	-	+	0.20	0.20	0.40
	37	16.0	14.6	+			0.35	0.44	0.79
	38	16.1	14.4	+			0.30	0.30	0.60
	39	15.9	13.3	-	-	-	-	-	-
	40	15.9	13.4	-	-	+	0.20	0.20	0.40
	41	17.2	25.0	+			0.60	0.91	1.51
	42	17.2	24.5	+			0.60	0.75	1.35
	43	16.0	23.8	+			1.12	1.80	2.92
	44	16.1	25.0	+			0.60	0.75	1.35
85%	45	15.0	23.0	+			0.80	0.94	1.74
Humidity	46	15.0	24.0	+			0.80	1.00	1.80
	47	17.4	23.4	+			0.60	0.75	1.35
	48	17.3	23.2	+			0.60	0.75	1.35
	49	17.0	25.0	+			1.70	2.10	3.80
	50	17.1	24.8	+			1.70	1.90	3.60
	51	15.0	19.3	+			0.80	0.92	1.72
	52	15.1	19.2	+			0.80	0.80	1.60
	53	15.0	19.8	+			0.60	0.80	1.40
	54	15.0	18.6	+			0.60	0.80	1.40
98%	55	15.0	19.0	+			0.80	0.90	1.70
Humidity	56	15.0	19.7	+			0.90	1.00	1.90
	57	15.2	19.3	+			0.56	0.80	1.36
	58	15.3	20.2	+			0.80	0.92	1.72
	59	15.4	19.8	+			0.90	0.92	1.82
	60	15.4	20.0	+			0.90	0.90	1.80

(+) = Detectable
(-) = Not detectable
Obs.: The samples which were positive in 10 days were not analyzed after 20 and 30 days.

maize (7), with a production of toxins in 60% of the samples.

At 40°C and 96% RH it was observed a production of aflatoxins in 80% of the samples, 70% being after 10 days of incubation and 10% after 30 days. The high MC in the samples, post-incubation, and the relative humidity of the environment must have influenced positively the production of aflatoxins, since the temperature of 40°C is not considered favorable (5,6).

At 40°C and 85.5% of relative humidity there was no production of aflatoxins, a fact which can be attributed to other factors not evaluated in our experiment.

It was thus concluded that the lowest temperatures (15°C) with a relative humidity of 61% and MC 13.0% seem to be best conditions for the storage of maize.

RESUMO

Influência da temperatura e umidade relativa na produção de aflatoxinas em amostras de milhos armazenados e contaminados artificialmente com *Aspergillus flavus*.

Foram utilizadas 90 amostras de grãos de milho provenientes de silos de várias regiões do Brasil, para se verificar a influência da temperatura e da umidade relativa na produção de afla-

TABLE 4 - Moisture content of the grain and production of aflatoxins in 30 samples of maize from storage, inoculated with toxigenic *Aspergillus flavus* at 40°C and 61.5, 85.5 and 96% of relative humidity after 10, 20 and 30 days of incubation.

	Samples	Moisture pre incubation (%)	Moisture pos incubation (%)	Qualitative Analysis for aflatoxins 10	20 (Days)	30	Level of aflatoxins B1 (ppm)	G1	Total of B1 + G1 (ppm)
	61	12.1	11.4	-	-	+	0.03	0.04	0.07
	62	12.0	11.3	-	-	+	0.03	0.04	0.07
	63	12.0	11.4	-	-	-	-	-	-
	64	12.1	11.2	-	-	-	-	-	-
61.5%	65	14.0	12.1	-	-	+	0.03	0.03	0.06
Humidity	66	14.0	12.0	-	-	-	-	-	
	67	14.7	12.0	-	-	+	0.02	0.03	0.05
	68	14.7	12.0	-	-	+	0.05	0.06	0.11
	69	14.6	12.0	-	-	+	0.03	0.03	0.06
	70	14.7	12.1	-	-		-	-	-
	71	14.2	14.6	-	-	-	-	-	-
	72	14.2	14.5	-	-	-	-	-	-
	73	13.4	14.6	-	-	-	-	-	-
	74	13.4	14.6	-	-	-	-	-	-
85.5%	75	12.3	14.2	-	-	-	-	-	-
Humidity	76	12.3	14.1	-	-	-	-	-	-
	77	12.6	13.8	-	-	-	-	-	-
	78	12.0	13.7	-	-	-	-	-	-
	79	12.0	13.7	-	-	-	-	-	-
	80	12.0	13.8	-	-	-	-	-	-
	81	12.2	18.0	-	-	-			
	82	12.1	18.1	-	-	+	0.22	0.30	0.52
	83	13.4	18.6	+			0.22	0.30	0.52
	84	13.4	18.5	+			0.30	0.30	0.60
96%	85	12.1	18.8	+			0.40	0.50	0.90
Humidity	86	12.0	18.8	-	-		-	-	-
	87	13.0	18.7	+			0.50	0.50	1.00
	88	13.2	18.8	+			0.50	0.50	1.00
	89	13.4	18.6	+			0.40	0.50	0.90
	90	13.2	18.5	+			0.40	0.60	1.00

(+) = Detectable
(-) = Not detectable
Obs.: The samples which were positive in 10 days were not analyzed after 20 and 30 days.

toxinas neste substrato. As amostras foram inoculadas com *Aspergillus flavus* toxigênico e incubadas em ambiente com temperatura e umidade relativa controladas (15°C e 61% UR, 86.5% UR, 99% UR; 25°C e 64% UR, 85% UR, 98% UR; 40°C e 61.5% UR, 85.5% UR, 96% UR) por períodos de 10, 20 e 30 dias de incubação. Foram detectadas aflatoxinas nas amostras incubadas a 15°C e 86.5% UR; 25°C nas três condições de umidade estudadas; 40°C e 61.5% UR, 96% UR. As melhores condições para produção de aflatoxinas foram 25°C e 85% UR e 98% UR. Os menores teores foram obtidos a 40°C e 61.5% UR.

Conclui-se que temperaturas mais baixas (15°C) com umidade relativa de 61% e conteúdo de umidade de 13% parecem ser as melhores condições para armazenamento de grãos de milho.

Palavras-chaves: milho, milho armazenado, *Aspergillus flavus*, aflatoxinas, temperatura, umidade relativa, umidade.

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SELECTION AND CHARACTERIZATION OF *RHIZOBIUM* SPP. STRAINS STABLE AND CAPABLE IN FIXING NITROGEN IN BEAN (*PHASEOLUS VULGARIS* L.)

Nadja M. Horta de Sá[1]
Maria Rita M. M. L. Scotti[1]
Edilson Paiva[2]
Avílio A. Franco[3]
Johanna Döbereiner[3]

SUMMARY

The variability in N_2 fixation effectiveness was determined by acetylene reduction tests of nodules formed by strains of *Rhizobium* spp before and after exposure to high temperatures. Nodules formed by strains more tolerant to high temperatures (*R. leguminosarum* bv. *phaseoli* and *R. tropici*) did not suffer any alterations in nitrogenase activity, plant dry weight and total plant nitrogen fixed. The protein pattern (eletrophoresis SDS-PAGE) differenciated strains among and within both species. The DNA hybridization using a nif probe marked through nick translation (biotin 14 dATP), when total DNA was digested with *Eco* RI, differenciated species of *R. leguminosarum* bv. *phaseoli* from *R. tropici* Polymorphism among the strains of *R. leguminosarum* bv. *phaseoli* after digestion with *Bam* HI and among *R. leguminosarum* bv. *phaseoli* and *R. tropici* after digestion with *Hind* III were also observed. Exposure to high temperature did not affect the protein or genomic patterns or nitrogenase activity. This may indicate that strains from both species (*R. leguminosarum* bv. *phaseoli* and *R. tropici*) that are tolerant to high temperature are also more genetic stable.

Key words: *Phaseolus vulgaris* L., *Rhizobium* termo tolerant, nitrogenase activity, protein and genomic patterns.

INTRODUCTION

Bacteria of the genus *Rhizobium* interact with legumes eliciting a simbiotic process, where nitrogen fixed by the bacterium is assimilated by the plant. However, it has been frequently observed that the strains of *Rhizobium leguminosarum* biovar *phaseoli* used in bean inoculation, present nodulating and/or simbiotic variability. On the other hand, the strains of *Rhizobium* that nodulate bean have been described as belonging to an heterogeneous group according to different criteria of characterization like protein patterns (9), antibiotic resistance (1), serological groups (18), multilocus

1. Departamento de Botânica - Instituto de Ciências Biológicas da UFMG - Cidade Universitária - 31270-901 - Belo Horizonte - MG - Brasil
2. EMBRAPA - CNPMS - 35700-000 - Sete Lagoas - MG - Brasil
3. EMBRAPA - CNPBS - 23851-970 - Seropédica - RJ - Brasil

enzyme eletrophoresis (17), DNA - DNA hibridization patterns (7) and plasmid profiles (12).

The genetic information which controls the symbiotic activity of *Rhizobium* strains nodulating beans is located on plasmids (12). These symbiotic plasmids (Psym) hold from one copy to several copies of the gene coding for nitrogenase (nif genes). The *Rhizobium* strains containing multiple copies of nif genes on plasmids have a narrow nodulation host range and were previously distinguished as type I, while those containing single nif genes copies on the plasmids, nodulate bean and *Leucaena* spp. and were distinguished as type II (10). Recently Martinez et al. (13), on the basis of the results of genetic analysis and phenotypic characteristics, considered the type II strains as a new species - *Rhizobium tropici*.

The reiterated regions of the nif genes seem to be normally necessary for the whole expression of the symbiotic effectiveness (19) and they can represent sites of recombinations producing genomic rearrangements which constitute the molecular basis of the variability and loss of the symbiotic properties in these strains of *Rhizobium* (4, 21, 24). Flores et al. (4) have analysed direct descendents of a single cell of *R. leguminosarum* bv. *phaseoli* after sucessive cultivations under normal laboratory conditions and in some cases, when they used recombining plasmids pMF101 and pMF122 as probes, they have detected alterations in their genome in relation to the patterns of the original cells.

The genetic instability of *R. leguminosarum* bv. *phaseoli* presents a serious problem for inoculum production. This factor is agravated in tropical regions where stress conditions, such as high temperatures, can favor the instability through rearrangements (4, 21) or curing of plasmids (11, 25). On the other hand, Hungria & Franco (6) have observed high levels of nitrogen fixation of beans grown at temperature above 38°C when inoculated with rhizobia isolated from *Leucaena*.

Aiming at selecting strains of *Rhizobium* efficient and stable in nitrogen fixation in beans, experiments were developed in order to compare variability of nodulation and N_2 fixation, as well as protein and genomic patterns before and after growing several generations of each strain at the highest temperature they were able to grow.

MATERIALS AND METHODS

Experiment I - Nineteen strains of *Rhizobium* spp. from "Centro Nacional de Pesquisas em Biologia do Solo" (CNPBS) and the "Centro de Pesquisas Agropecuárias dos Cerrados" (CPAC) collections, were tested for their N_2 fixation capacity in *Phaseolus vulgaris* L. cv. Negro Argel in Leonard jars (23). Inoculation (2 seeds/jars) was performed with 2 ml of cells grown in yeast mannitol medium (YM) to the final logarithmic phase. Three repetitions were used, and a treatment were *Leucaena* was used as a host plant was also included. After 28 days of growth, the plants were harvested and 10 nodules/plant of uniform size (fresh weight between 5.0 and 7.0 mg), chosen at random, were detached from the roots and placed in flasks hermetically closed, where 10% of the atmosphere was substituted by acetylene. After incubation for 10 minutes the ethylene produced was measured using a gas chromatograph (Variant - 2440, flame ionization detector, column poropak N, carrier N_2).

Experiment II - Determination of the maximum temperature for growth - selection "*in vitro*" of clones growing at high temperatures.

Isolated colonies of each strain were grown up to the final log phase (10^8 cells/ml) in YM at 29°C. For determining the maximum temperature of growth for each strain, transfers from the initial growth were made, corresponding each inoculum to 1% of the total volume of the medium and incubated on a shaker at 29°C (optimum temperature) and at 35, 36, 37, 38, 39, 40, 41 and 42°C. The strains were grown again twice at the maximum temperature they would grow up to the final log phase and stored thereafter at 4°C.

Experiment III - Six strains, the most tolerant to high temperature, after exposure to their maximum growth temperature, as well as the parent strains, were tested and compared for their variability of N_2 fixation after analysis by acetylene reduction (ARA) in the same condition of Experiment I. Subsequently isolations from 10 nodules of each plant were performed according to Vincent (23). The dry weight of the plants was determined after drying at 65°C for 48 hours and its nitrogen content determined by microkjeldahl method (22).

Experiment IV - Protein pattern determinations. SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel eletrophoresis was conducted according to Laemmli (8) with modifications as described below. Wild type strains (especially the most tolerant to high temperature) of the *R. leguminosarum* bv. *phaseoli* and *R. tropici*, both before and after growth at high temperature, and their isolates from nodules of different levels of

ARA were grown in YM up to the beginning of the stationary phase, corresponding approximately to 10^9 cells/ml. The cells were centrifugated (8000 x g for 10 min at 4°C), washed and suspended in buffer PBS pH 7.4. Then they were disrupted through sonication (sonifier Branson, output-3, duty cycle 75%) for 4 min at intervals of 2 min. Desnaturized samples which were obtained by 5 min heating, in buffer Tris 62.5 mM, 2.3% SDS, pH 6.8 added of mercaptoetanol 5% were used. The amount of protein added to each well was 18 mg. The protein concentration was determined according to the procedure described by Bradford (2). The concentration of the acrylamid gradient used, varied from 7.5 to 17.5%. The running buffer used was the Tris-glycine with SDS (3.03 g Tris HCl; 14.41 g glycine and 1.0 g SDS per litre). The samples were submited to an initial current of 12 mA followed by application of 24 mA after penetration on the stacking-gel. At the end of the eletrophoresis the gel was dyed with comassie blue 0.2%.

Experiment V - Genomic pattern determinations. Wild type strains, especially the most tolerant to high temperature, after exposure to their maximum growth temperature, and their isolates from nodules of different levels of ARA, were grown in YM up to the log phase (10^8 cells/ml). The total DNA of the strains was isolated, using Hahn & Hennecke's method (5) with the following modification. After precipitation of the DNA, by addition of 1/10 vol 5M NaCl and 2 volumes ethanol (-20°C) it was directly transferred to 76% ethanol, 0.2 M NaOAC and then resuspended and allowed to stay overnight in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0).

After spectrophotometric padronization, the total DNA was digested by the restriction enzymes *Eco* RI, *Hind* III and *Bam* HI and submited to eletrophoresis in agarose gel 0.6% 30 volts/16mA for 18 hours. Then, the DNA was transferred to membranes of nitrocellulose, according to Sambrook et al. (20). The probe used was a plasmid recombinant; pcQ15 which carries a 4.7 Kilobase *Eco* RI insert with nitrogenase structural genes (nif genes). The probe was marked through nick translation in the presence of 14 dATP-biotin. The labelling was made according to the description of the DNA Detection System Instruction Manual - BRL, being the biotinilated probe separated in sephadex G-50 columns. The hybridization procedure of the DNA was done according to Medeiros et al. (14) at the temperature of 42°C for 18 h and the final detection of the probe connection to the DNA target was done according to the Blue Gene Kit of BRL - non radioactive nucleic acid detection system.

RESULTS AND DISCUSSION

Determination of the nitrogen fixation variability of Rhizobium *strains capable of nodulating bean.* - Among the nineteen strains tested, all nodulated bean, six did not nodulate *Leucaena* (*R. leguminosarum* bv. *phaseoli*) and thirteen nodulated both bean and *Leucaena* (*R. tropici*) (Tab. 1). The acetylene reduction activity evidenced a great variability among individual nodules of the same strain (data not show), following a normal distribution, similarly to what was found with soybean nodules by Peres et al. (16). Differences were also observed in the mean activities of nodules of each strain (Tab. 1). Three strains of each species were able to grow at temperatures equal or superior to 38°C (tab. 1). These strains more tolerant to heat, after growing twice at the maximum temperature (38 or 39°C), were grown at 29°C and were inoculated into beans for comparison of their N_2 fixation activities with their parent strains. High temperatures, among other stress conditions, can increase the frequency of alterations verified in the characteristics of effectiveness of N_2 fixation in *Rhizobium* capable of nodulating bean (21). However in the present experiment acetylene reduction activity of the strains more tolerant to heat from both species of *Rhizobium* were not altered. The same was also verified for plant dry weight and total plant nitrogen (tab. 2). The distribution of the individual nodule activity of these strains (Fig. 1) was quite similar, except as CPAC H_{14} (*R. leguminosarum* bv. *phaseoli*) and CENA CO_5 II (*R. tropici*) which presented less homogeneous distributions when compared to the parental strains. However, their mean activities did not differ significantly either (table 2).

Protein patterns - The comparison of the protein patterns showed differences both among the strains of *Rhizobium* of the same species and between the species. The strains of *R. tropici* presented more homogeneous profiles while in *R. leguminosarum* bv. *phaseoli* differences were evident in a great number of polypeptides (Fig. 2). Strains of both species, when submitted to high temperatures, did not present alterations in their protein patterns in relation to the parent strain (Fig. 3). Isolates of nodules of different levels of

TABLE 1 - Nodulation of bean (*Phaseolus vulgaris* L.) and *Leucaena leucocephala* and acetylene reduction of individual nodules inoculated with 6 strains of *R. leguminosarum* bv. *phaseoli* and 13 strains of *R. tropici* as well as their maximum growth temperature.

Strains	Bean Nodulation	*Leucaena* Nodulation	Maximum growth temperature (°C)	Nitrogenase activity (a) (nmoles C_2H_4/h/nodule)
R. leguminosarum bv. *phaseoli*				
Br 365 (CNPAF 146)	+	–	37	104.13
Semia 476 (CPAC H_{19})	+	–	38	91.01
CPAC H_{30} (IPAGRO 1102)	+	–	37	62.02
CPAC H_{35} (IPAGRO 1378)	+	–	39	48.42
CPAC H_{23} (V_{23} RGS)	+	–	37	37.80
CPAC H_{14}	+	–	39	29.75
R. tropici				
CIAT 899 (Br 322)	+	+	38	132.38
UFP 491 (CPAC H_{21})	+	+	37	125.38
CPAC H_{20}	+	+	38	105.02
Na 82 (Br 10.013)	+	+	37	80.55
Br 817 (NGR8)	+	+	36	51.56
Br 818 (TAL1145)	+	+	35	45.06
CENA CO_5 II (Br 266, Semia 492)	+	+	39	36.86
Car 22 (Br 10.014)	+	+	37	30.01
CPAC H_{36}	+	+	37	27.83
USA 1070 (CPAC H_{38})	+	+	36	22.48
CPAC H_{26} (IPAGRO 1020)	+	+	36	22.30
Br 814 (DF10)	+	+	36	14.75
CFN 299	+	+	<35	7.04

(a) Means of 60 nodules/strain.

TABLE 2 - Mean activity of acetylene reduction in 60 bean nodules produced by strains of *R. tropici* and *R. leguminosarum* before and after growth at high temperatures, as well as plant dry weight and total plant N.

Strains	Growth temperature (°C)	Acetylene reduction (nmoles C_2H_4/h/nodule)	Plant dry weight (g/plant)	Total Plant N (mg/plant)
CPAC H_{20} (a)	29	58.75	0.61	142
CPAC H_{20} (b)	38	50.02	0.58	110
CENA CO_5 II (a)	29	38.14	0.74	180
CENA CO_5 II (b)	39	39.30	0.70	161
CIAT 899 (a)	29	107.30	0.82	171
CIAT 899 (b)	38	105.73	0.85	180
CPAC H_{14} (b)	29	36.6	0.52	61
CPAC H_{14} (b)	39	32.7	0.43	52
Semia 476 (a)	29	40.10	0.66	159
Semia 476 (b)	38	35.32	0.53	120
CPAC H_{35} (a)	29	32.40	0.44	122
CPAC H_{35} (b)	39	35.15	0.34	91

(a) Parent strain and (b) strains grown twice in the temperature indicated. No significant differences (Ducan, 5% of probability) in the activities of acetylene reduction, dry weight and total N were observed.

acetylene reduction activity of a strain, did not show any variation in protein patterns either (data not shown).

Genomic patterns - The results of hybridization of the total DNA of the parent strains of *Rhizobium*, after digestion with *Eco* RI, showed differences among the DNA patterns between strains of *R. leguminosarum* bv. *phaseoli* and *R. tropici*, but not among strains of the same species. Confirming the information of Martinez et al. (12, 13) that the strains of *R. leguminosarum* bv. *phaseoli* present multicopies of the nif genes while those of *R. tropici* present only one copy (Fig. 4). However, when the total DNA of these strains were digested with *Bam* HI (Fig. 5), the DNA of the strains of *R. leguminosarum* bv. *phaseoli* present-

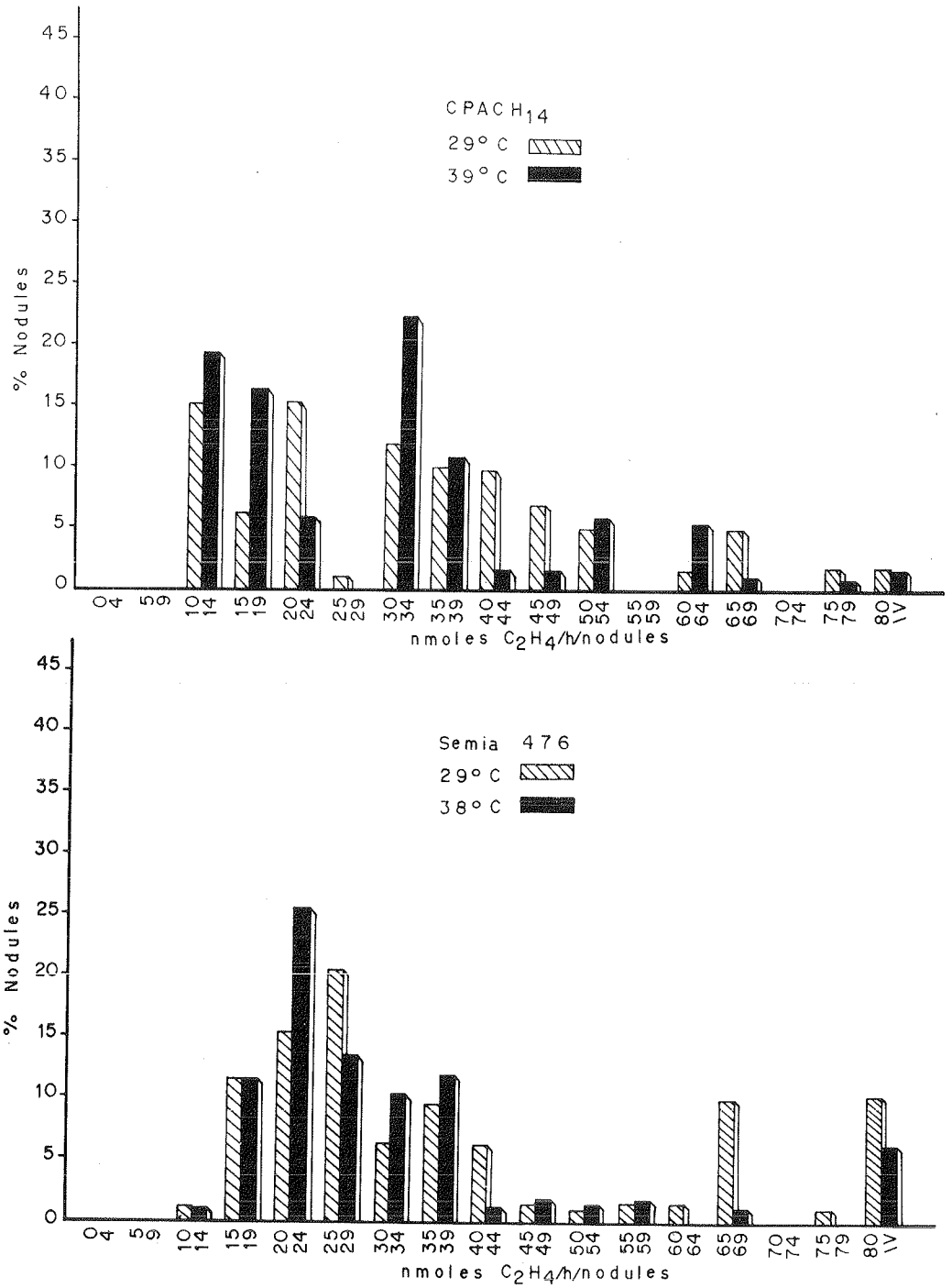
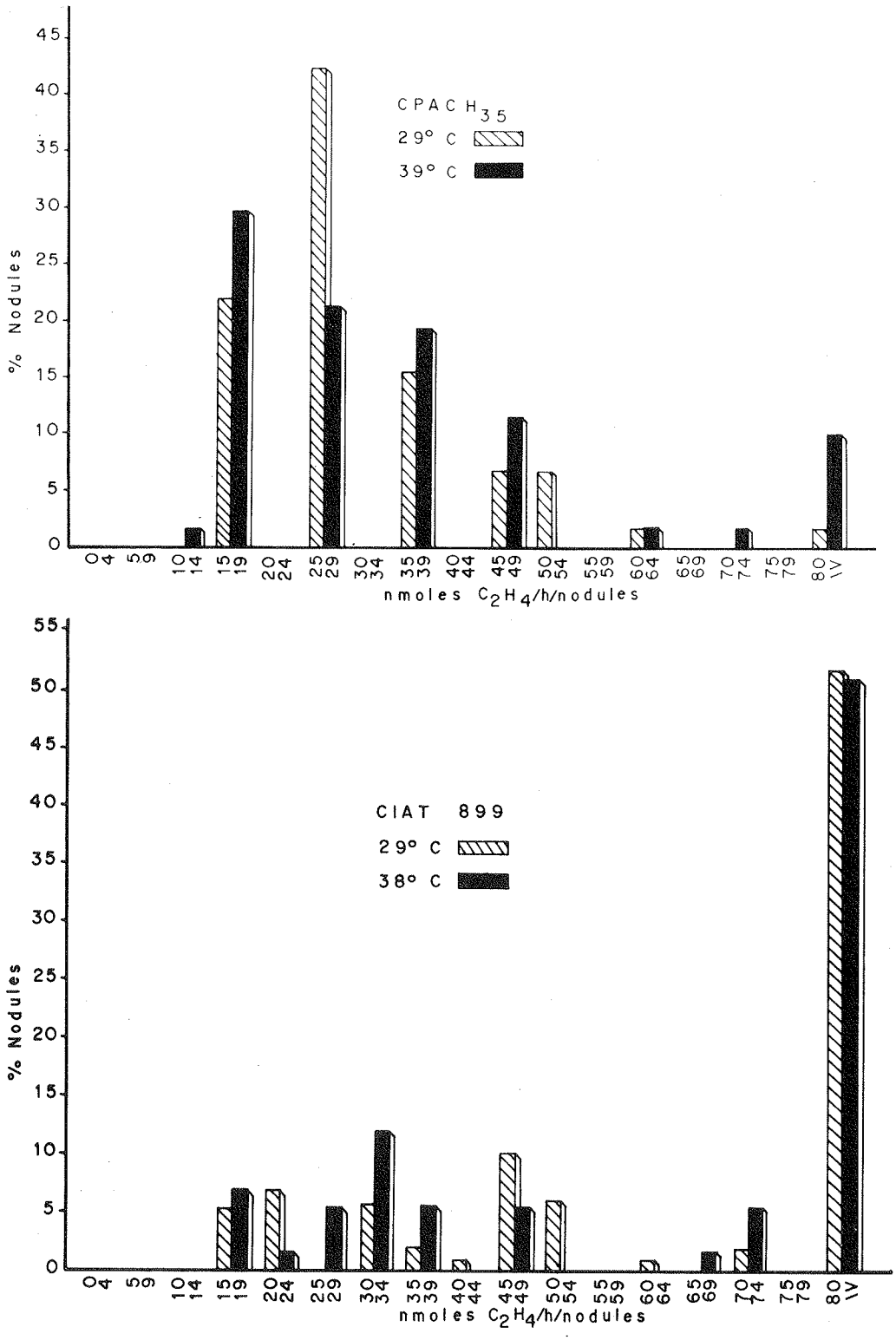
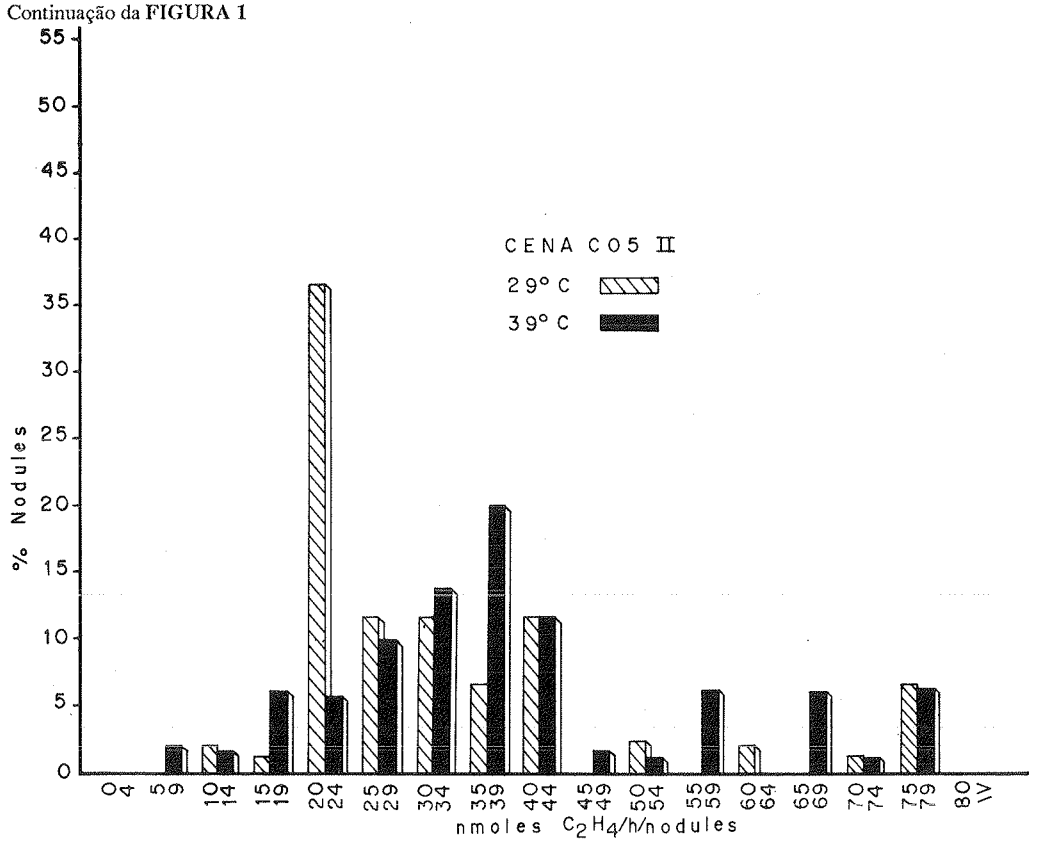


FIGURE 1 - Activity of acetylene reduction in 60 individual nodules of bean after inoculation with *R. leguminosarum* bv. *phaseoli* strains: Semia 476 parent (29°C), Semia 476 exposed to high temperature (38°C); CPAC H_{14} parent (29°C), CPAC H_{14} exposed to high temperature (39°C); CPAC H_{35} parent (29°C), CPAC H_{35} exposed to high temperature (39°C) and *R. tropici* strains: CIAT 899 parent (29°C), CIAT 899 exposed to high temperature (38°C); CENA CO_5 II parent (29°C), CENA CO_5 exposed to high temperature (39°C); CPAC H_2O parent (29°C), CPAC H_2O exposed to high temperature (38°C).

Continuação da **FIGURA 1**



Continuação da FIGURA 1

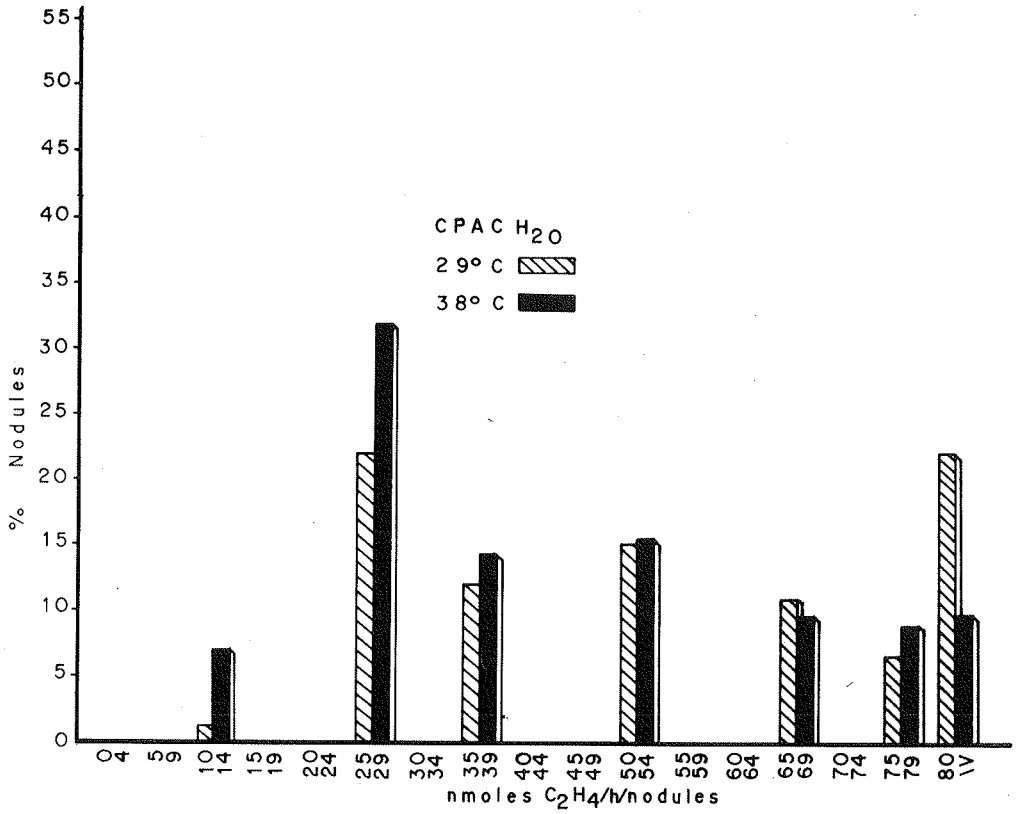


ed polymorphism and conservation of the central band in all of them. Strain Semia 476 presented a different pattern from the others; strains CPAC H_{14} and CPAC H_{35} presented identical patterns between them, however different from the others, similarly to what happened to strains Br 365 and CPAC H_{23}. Strains of *R. tropici*: CIAT 899, CENA CO_5 II and CPAC H_20 presented homologous patterns among them, with the presence of two hybridization bands in the same position. When the DNA was digested with *Hind* III, the polymorphism was much more evident. The strains presented specific cleavage patterns of the DNA indicating genetic variations at the level of their nucleotide sequencies (fig. 6). Among the strains of *R. leguminosarum* bv. *phaseoli*, strain Semia 476, similarly to what happened after digestion with *Bam* HI, also presented a different pattern from the others, strains Br 365 and CPAC H_{14} presented the same patterns between them, however different from the others, similarly to what happened to strains CPAC H_{35} and CPAC H_{23}. However, the strains of *R. tropici* after digestion with *Hind* III presented polymorphism not shown when digested with *Eco* RI and *Bam* HI.

Strains CIAT 899 and CENA CO_5 II presented the same pattern in their total DNA, but different from the others, as to what happened to strains CPAC H_{20} and Na 82. With *Hind* III, strains Br 814, Br 817 and Br 818 present different patterns among them, as well as in relation to the other strains tested.

In an attempt to detect modifications in the molecular characteristics of strains of *Rhizobium* after exposure to high temperature, the hybridization patterns of their total DNA with the nif probe were compared to their parent pairs, after digestion with *Eco* RI. The results, Fig. 7, show that there were no alterations in relation to the original patterns in the strains of both *Rhizobium* species, showing that the Sym plasmids of these strains were not modified or cured by *in vitro* growth at 38°C. This constrasts with results obtained by others (3, 11) who used strains that were not selected for tolerance to high temperature. The results of hybridization test with isolates of different levels

Continuação da **FIGURA 1**



of nitrogenase activities obtained from *R. leg.* bv. *phaseoli* (Strain Semia 476) and *R. tropici* (strain CIAT 899) did not show polymorphism in their total DNA with nif probe, after digestion with *Eco* RI (Fig. 8).

The differences evidenced both in the protein patterns (Fig. 2) and in the total DNA hybridization patterns (Fig. 4, 5, and 6) between species (*R. leg.* bv. *phaseoli* and tropici) and in the parent strains, confirm the data of the literature about heterogeneity of the strains of *Rhizobium* nodulating bean. However, our results suggest a diversification which is more evident in the strains of *R. leg.* bv. *phaseoli* than in those of *R. tropici*. The analysis of the more homogeneous protein patterns of *R. tropici*, rather than those of *R. leg.* bv. *phaseoli* (Fig. 2)

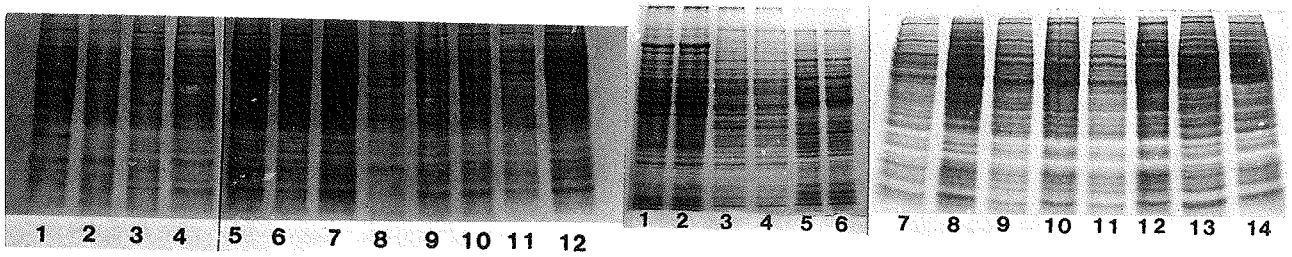


FIGURE 2 - Eletrophoresis SDS-PAGE. Total protein of patterns of *R. leguminosarum* bv. *phaseoli* (1) Semia 476, (2) CPAC H_{35}, (3) CPAC H_{14}, (4) Br 365 and of *R. tropici:* (5) ciat 899, (6) cena cos II, (7) CPAC H_2O, (8) Car 22, (9) Br 814, (10) Br 818, (11) Br817, (12) Na 82.

Figure 3 - Eletrophoresis SDS-PAGE. Total protein patterns of *Rhizobium* spp.: (1) CPAC H_{35} º, (2) CPAC H_{35}*, (3) CPAC H_{14} º, (4) CPAC H_{14}*, (5) Semia 476º, (6) Semia 476*, (7) CPAC H_{20}º, (8) CPAC H_{20}*, (9) CIAT 899º, (10) CIAT 899º, (10) CIAT 899*, (11) CENACO $_5$IIº, (12) CENACO $_5$II*, (13) Car$_{20}$º, (14) Car$_{22}$*, º - parent strain,
* Strain grown twice at maximum growth temperature.

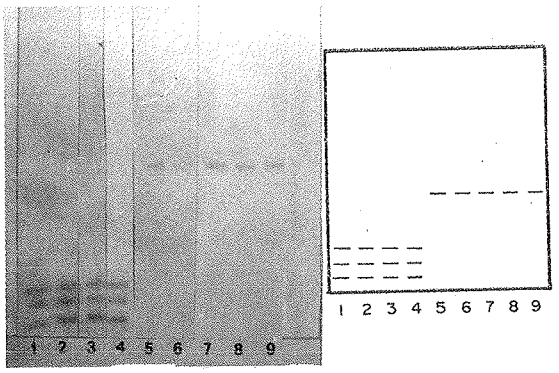


FIGURE 4 - Southern blot hibridization, nif probe marked with biotine through nick translation. Total DNA digested with *Eco* RI of the original strains of *R. leg.* bv. *phaseoli* (1) Semia 476, (2) CPAC H_{14}, (3) CPAC H_{35}, (4) CPAC H_{23} and of *R. tropici*, (5) CIAT 899, (6) CENA CO_5 II, (7) H_{20}, (8) Br 817, (9) Na 82.

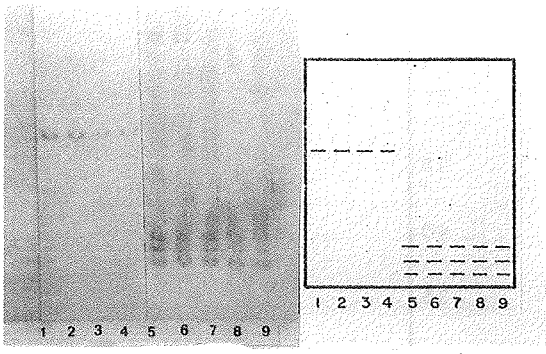


FIGURE 7 - Southern blot hibridization, nif probe marked with biotine through nick translation. Total DNA digested with *Eco* RI of the strains (1) Semia 476 º, (2) Semia 476*, (3) CPAC H_{35} º, (4) CPAC H_{35}*, (5) CPAC H_{14} º, (6) CPAC H_{14}*, (7) CIAT 899 º, (8) CIAT 899*, (9) CENA CO_5 II º, (10) CENA CO_5 II*, (11) CPAC H_{20}º, (12) CPAC H_{20}*.

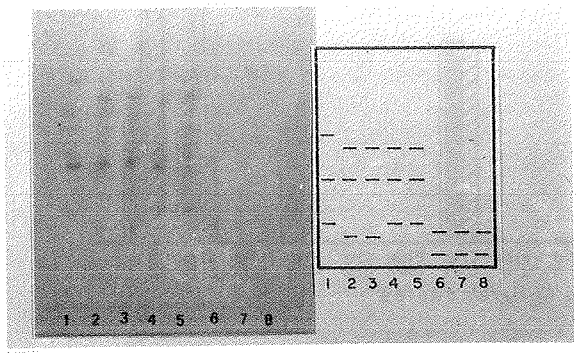


FIGURE 5 - Southern blot hibridization, nif probe market with biotine through nick translation. Total DNA with *Bam* HI of the original strains of *R. leg.* bv. *phaseoli* (1) Semia 476, (2) CPAC H_{14}, (3) CPAC H_{35}, (4) Br 365, (5) CPAC H_{23} and of *R. tropici*, (6) CIAT 899, (7) CENA CO_5 II, (8) CPAC H_{20}.

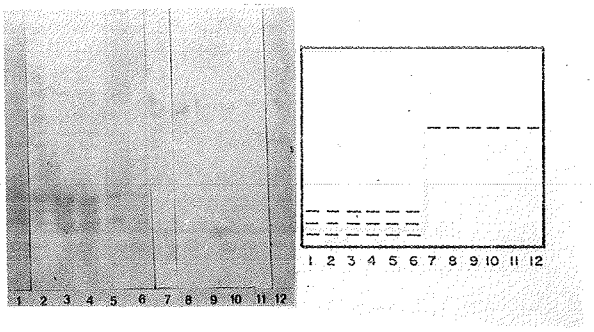


FIGURE 8 - Southern blot hibridization, nif probe marked with biotine through nick translation. Total DNA digested with *Eco* RI of isolates from nodules with different levels of effectiveness of CIAT 899 (1 low, 2 medium, 3 and 4 high effectiveness) and Semia 476 (5 and 6 low, 7 medium 8 and 9 high).

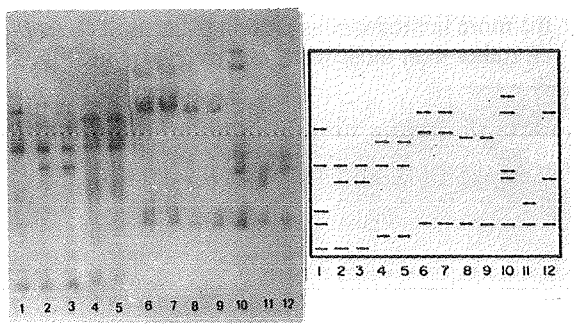


FIGURE 6 - Southern blot hibridization, nif probe marked with biotine through nick translation. Total DNA digested with *Hind* III of the original strains of *R. leguminosarum* bv. *phaseoli* (1) Semia 476, (2) Br 365, (3) CPAC H_{14}, (4) CPAC H_{35}, (5) CPAC H_{23} and of *R. tropici*, (6) CIAT 899, (7) CENA CO_5 II, (8) CPAC H_{20}, (9) Na 82, (10) Br 814, (11) Br 817, (12) Br 818.

and the tests of the DNA hybridization, where a greater number of hybridization bands was found, as well as a more acentuated polymorphism among the strains of *R. leg.* bv. *phaseoli* show a greater genetic variation in these strains. Recent results from Moreira et al. (15) indicate that fast growing rhizobia show greater variability in protein pattern than the strains of slow growth. *R. tropici* seems to be a link between the typical fast growing *R. leguminosarum* bv. *phaseoli*, specific for bean and the more promiscuous *Bradyrhizobium* which also nodulates *Leucaena*, a species which is nodulated by fast and slow growing rhizobia (13).

Flores et al. (4) and weaver et al. (24) showed that, in the majority of the cases, genetic studies of *R. leguminosarum* bv. *phaseoli* developed after cultivation through several generations

and submitted to hybridization tests with various kinds of probes and comparison of plasmid profiles did not present genome differences in relation to their original clones. Only in some cases was it possible to show some kind of alterations. These observations show the difficult in detecting molecular alterations in strains exposed to high temperature or among isolates of the same strain showing different levels of nitrogenase activity. The alterations resulting from genetic rearrangements are probably confined to certain regions of the genome (4) and, having in mind this perspective, additional experiments would be necessary, as for example the use of other probes to detect them. It is also possible that the lack in detection of alterations in the strains submitted to high temperature actions is a result of the previous selection of the strains tolerant to high temperature and may represent an effective procedure to identify more genetic stable strains. All these studies could contribute to the understanding of the frequent alterations verified in the symbiotic characteristics of the strains of *Rhizobium* that nodulate beans. On the other hand, the selection and characterization of strains toleranting high temperatures and capable of maintaining their relevant characteristics in these conditions could represent a promising alternative for the bean inoculation in tropical soils.

RESUMO

Seleção e caracterização de estirpes de *Rhizobium* estáveis e capazes de fixar nitrogênio em feijão (*Phaseolus vulgaris* L.)

Determinou-se a variabilidade na capacidade de fixação de N_2, através de testes de redução de acetileno em nódulos formados por estirpes de *Rhizobium*, antes e após exposição das bactérias "*in vitro*" à temperaturas elevadas (38-39°C). Nódulos formados tanto por estirpes de *R. leguminosarum* bv. *phaseoli* como *R. tropici* mais tolerantes a altas temperaturas, quando inoculadas em feijão, não sofreram alterações nas características simbióticas tais como, atividade de nitrogenase, peso seco de planta e nitrogênio total fixado. O padrão de proteínas (eletroforese SDS-PAGE) diferenciou estirpes entre e dentro das espécies. A hibridização do DNA total usando "nif probe" marcada via "nick translation" (biotina 14 dATP), quando a digestão foi efetuada com *Eco* RI, diferenciou a espécie de *R. leguminosarum* bv. *phaseoli* de *R. tropici*. Foi observado polimorfismo entre as estirpes de *R. leguminosarum* bv. *phaseoli* após digestão com *Bam* HI e entre *R. leguminosarum* bv. *phaseoli* e *R. tropici* após a digestão com *Hind* III. Não foram detectadas alterações nos padrões protéicos ou genômicos e na atividade da nitrogenase da mesma estirpe antes e após crescimento a temperaturas elevadas, indicando que as estirpes de ambas as espécies (*R. leguminosarum* bv. *phaseoli* e *R. tropici*), tolerantes a altas temperaturas são também mais estáveis geneticamente.

Palavras-chave: *Phaseolus vulgaris* L., *Rhizobium* termo-tolerantes, a atividade da nitrogenase, padrões protéicos e genômicos.

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FUNGI FROM SOIL AFFECTED BY BIRDS IN THE "PARQUE ESTADUAL DAS FONTES DO IPIRANGA", SÃO PAULO STATE, BRAZIL

Andrea Ninomiya
Marli de Fátima Rodrigues Antunes
Iracema Helena Schoenlein-Crusius

SUMMARY

The possible effects of the presence of birds on some microbiological and abiotic parameters of the soil were studied. Five soil samples were taken monthly at 15cm depth from five sites affected by the presence of the bird and control sites from the margins of a pond in "Parque Estadual das Fontes do Ipiranga", São Paulo, Brazil. Fungi were isolated by the soil plate method combined with baiting techniques using cellulosic, keratinic and chitinic substrates. Thirty taxa of fungi were isolated and identified, being 22 of Deuteromycotina (Hyphomycetes), 01 of Ascomycotina (Plectomycetes) and 07 of Mastigomycotina (03 of Oomycetes and 04 of Chytridiomycetes). The means of air and soil temperature were not significantly different in the sites. pH and moisture content were significantly lower in the soil samples taken from the affected sites. The total number of fungal colonies was higher in the affected soil, but the fungal diversity was lower, indicating the occurrence of restrictive conditions of the environment for the development of the native mycota.

Key words: birds, soil mycota, pond.

INTRODUCTION

Deforestation in the State of São Paulo has been the reason for the search of migratory birds for new refuges. This has caused a decrease of the population of some tree species, particularly in recently colonized ecosystems. This has been observed in a forest located by the "Lago das Garças" pond in the "Parque Estadual das Fontes do Ipiranga", managed by the Botanical Institute in São Paulo, SP. The increase of the bird populations has been disproporcional to the capacity of the forest to support the great number of animals such as *Botaurus pinnatus* (Wagler), *Phalacrocorax olivaceus* Brisson, *Egretta thula* (Gm.) Linn. and *Egretta alba* (Gm.) Linn. (7, 15, 24). The community structure of the vegetation has been changed in function of the excess of birds excrements introduced in the soil, especially since 1990. Our objective was to detect modifications of the soil mycota resulting from the presence of the birds.

MATERIALS AND METHODS

This experiment was realized in the Parque Estadual das Fontes do Ipiranga, SP, in the Instituto de Botânica. It is located at 22°39' latitude south and 46°37' longitude west and 798m altitude. The vegetation was classified as subtropical upland forest and, based on the International System of Köppen, the climatic conditions are of Cwb

Instituto de Botânica. Seção de Micologia e Liquenologia, Caixa Postal 4005, 01061-970 São Paulo, SP, Brazil.

type, mesothermic, with dry winters, anual rain volume over 1000mm and mean air temperatures between 18 to 22°C (22). Nutrient levels of the affected soil are higher than in the nonaffected sites, especially for phosphorus, which levels reached more than 1.000 ppm in the affected soil by birds dung (I. H. Schoenlein-Crusius, unpublished data).

Collection points were chosen in the effected areas and control sites were chosen in a nonaffected region, located about 50 m from that affected by the birds. Five 300 g soil samples were collected monthly from both areas between October 1990 and March 1991 from each site. The collections were always made between 13 to 14h to avoid effects of daily microclimatic fluctuations. Soil temperature, at 15cm deepth, and air temperature were measured in the field. Soil moisture (12) and pH were measured in the laboratory.

For fungal isolations, soil plate method was used with modified Martin's medium (25). Petri dishes were incubated at room temperature (22-27°C) during one week. Aquatic fungi were baitened with corn straw, hair, cellophane, snake skin, shrimp shell, pollen and *Sorghum* seeds (2, 13). The colonies were quantified and identified after one week, except for the zoosporic fungi, that were observed after five days. Identifications were conducted using special literature for each taxonomic group. The results were estatistically treated by the Student's T test (19).

RESULTS AND DISCUSSION

1. *Fungi* - The mean number of colinies of fungi in the bird affected soil (Table 1) ranged between 172,40 in October and 63,5 in March and varied between 104,00 in November and 48,71 in March in the control sites. The means of the total number of fungi isolated from the bird affected soil were statistically higher than the ones determinated in the control sites in October (T= 3.064; α = 1 and 5%), December (T= 2,23; α = 5%) and February (T= 2,21; α = 5%). Negative correlations between the number of fungi and fungal diversity were also found in the soil of Wadi Gena in Africa (1) . We identified 30 fungal species including 22 of Deuteromycotina (Hyphomycetes), 01 of Ascomycotina (Plectomycetes), 07 of Mastigomycotina, being 03 of Oomycetes and 04 of Chytridiomycetes (Table 2). Thirty nine total occurrences of fungi were registrated in the bird affected soil and fifty three total occurrences of fungi in the control soil. Yeasts were not isolated in the experiments. Results about the isolation and distribution of the Zygomycotina species obtained in the bird affected and control soil are going to be published in a next paper. The dominance of Imperfect Fungi in the soil mycota has been often mentioned and has also been reported in amended soils in tropical ecosystems (5, 23). Similar results concerning fungal distribution were observed in the rhizosphere soil of sugarcane crops in Pernambuco, where 89% of the fungi were Deuteromycotina, 9,5% Zygomycotina, 0,7% Ascomycotina and 0,8% Mycelia sterilia. *Penicillium, Aspergillus, Fusarium* and *Trichoderma* were also frequently isolated from the soil (18). Species of Deuteromycotina have presented a high capacity to produce enzymes, spores and efficient dispersal mechanisms in addition to a great colonization ability of a large range of organic compounds (8).

These results indicate possible negative correlations between fungal diversity and the number of colonies and can be justified by the changed conditions of the bird affected soil, establishing a selective effect on the fungal community. The species of fungi that resisted the high amount of birds excrements probably increased in number, justifying the high total colony numbers in the affected soil.

TABLE 1 - Means of the parameters determinated in the soil affected by the birds (AF) and control sites (CN), from October 1990 to March 1991.

Months	fungal colonies		temperature (C) air		soil		Soil pH		Soil moisture (%)	
	AF	CN	AF	CN	AF	CN	AF	CN	AF	CN
Oct. 90	172.40	99.60	34.00	33.00	20.00	20.00	4.38	5.70	—	—
Nov. 90	102.09	104.00	18.00	18.00	18.10	17.50	5.44	5.77	66.73	78.28
Dec. 90	126.00	88.53	29.80	27.80	21.30	23.60	4.11	5.26	68.28	84.18
Jan. 91	71.87	64.50	27.00	27.00	20.90	21.60	3.99	5.63	69.10	80.77
Feb. 91	87.38	57.40	20.00	22.70	19.50	19.40	4.44	5.04	58.97	77.68
Mar. 91	63.50	48.71	29.00	28.60	20.70	20.60	4.84	5.86	60.57	68.91

TABLE 2 - Occurences of the fungi isolated in six collections (Oct/1990 - Mar/1991) of soil from bird affected (AF) and control soils (CN).

Fungi	1990 Oct		Nov		Dec		1991 Jan		Feb		Mar		Total	
	AF	CN	AF	CN	AF	CN	AF	CN	AF	CN	AF	CN	AF	CN
Deuteromycotina														
Hyphomycetes														
Acremonium larvarum (Fetch) W. Gans.						X								1
Fusarium oxysporum Schlecht.									X				1	
emend. Snyd. & Hans.														
Fusarium sambucinum Fuckel.	X												1	
Fusarium solani (Mart.)			X										1	
Appel & Wollenw. emend. Snyd. & Hans.														
Penicillium crustosum Thom.							X						1	
Penicillium glabrum (Wehmer) Westling.			X										1	
Penicillium granulatum Bain.			X										1	
Penicillium implicatum Biourge.									X		X		2	
Penicillium islandicum Sopp.										X				1
Penicillium oxalicum Currie & Thom.		X		X		X				X				4
Penicillium spinulosum Thom.									X		X		2	
Penicillium verrucosum Dierckse.									X	X			1	1
Penicillium viridicatum Westling.						X		X		X				3
Trichoderma aureoviride Riffai.				X			X						1	1
Trichoderma hamatum (Bon) Bain.					X	X							1	1
Trichoderma harzianum Riffai.				X					X				1	1
Trichoderma koningii Oud.												X		1
Trichoderma piluliferum Webster & Riffai.									X				1	
Trichoderma pseudokoningii Riffai.										X	X		1	1
Trichoderma reesei Riffai.										X				1
Trichoderma viride Pers ex Gray	X		X	X	X	X	X	X	X	X		X	5	5
Verticillium fungicola (Preuss) Hasseir.					X	X			X				2	1
Ascomycotina														
Plectomycetes														
Eupenicillium sp.				X			X						1	1
Mastigomycotina														
Oomycetes														
Achlya flagellata Coker.	X	X	X	X	X	X		X		X			3	5
Achlya sp.		X												1
Saprolegnia parasitica Coker.						X				X		X		3
Chytridiomycetes														
Catenophlyctis variabilis karling.		X				X		X		X		X		5
Karlingia rosea (Karling) Karling.	X	X	X	X	X	X	X	X	X	X	X	X	6	6
Nowakowskiella elegans Nowak.	X	X	X	X	X	X	X	X	X	X	X	X	6	6
Rhizophydium elyensis Sparrow.						X		X		X		X		4
Total	5	6	7	8	6	12	6	7	10	13	5	7	39	53

The affected soil could have supported the development of *Fusarium* species, *Penicillium crustosum, Penicillium glabrum, Penicillium granulatum, Penicillium implicatum* and *Penicillium spinulosum* that occurred only in the soil affected by the birds. On the other hand, the affected soil could decrease the development of *Penicillium islandicum, Penicillium oxalicum* and *Penicillium viridicatum*, that were only isolated from the control sites. *Penicillium verrucosum* occurred in the affected and in the control sites. *Penicillium* and *Trichoderma* presented the highest number of species in the experiment. These genera are well adapted to different substrate conditions (16, 17), being frequently mentioned among the most common fungi in terrestrial and aquatic environments (14). The frequency of *Trichoderma* species were not affected by the presence of the birds excre-

ments. The strong cellulolitic ability of *Trichoderma* species is well known (11). *Trichoderma viride* was the most frequently isolated species in both studied areas. *T. viride* is mentioned to have the capacity to decrease the growth of other fungal taxa by the production of antibiotics (4).

Although the Ascomycetes normaly had presented high cellulolitic capacity and resistance against temperature changes in compost (9), their teleomorphs were represented in this study only by *Eupenicillium*, perfect state of *Penicillium*. *Karlingia rosea and Nowakowskiella elegans* frequencies were not affected by the presence of the bird excrements and occurred in all collections. *Catenophlyctis variabilis, Rhizophydium elyensis, Saprolegnia parasitica* and *Achlya* sp. occurred only on the control sites. Oomycetes and Chytridiomycetes are considered truly aquatic fungi because of their necessity of water for reproduction and dispersal mechanisms by zoospores (20). To guarant the survival of zoosporic fungi most of the species produce resistent structures and present the ability to grow in the soil water (6), justifying their presence in the birds affected site.

2. *Air and soil temperature* - In the affected areas the air temperature was higher in October, corresponding to 34°C. The lowest temperature of the air was registrated in November, corresponding to 18°C (Table 1). In the control sites the air temperature varied between 33°C (October) and 18°C (November). The soil temperature determinated at 15cm depth varied around 21,3°C in the affected area and 23,6°C in the control sites in December (Table 1). The lowest soil temperatures were determinated in November, corresponding 18,1°C in the affected area and 17,5°C in the control sites, not being estatistically different. The decomposition of great quantities of organic matter in the soil can cause an increase in the microbial metabolism and increases in the soil temperature, such as in composts (9).

3. *pH of the soil* - In the affected soil the pH varied between 3,99 and 5,44 (January and November, respectively), (Table 1). In the control sites the soil pH varied between 5,04 and 5,86 in February and March, respectively. Mean values of the pH in the soil were lower in affected areas in October (T= 2,36; α = 5%), January (T= 4,54; α = 1 and 5%) and March (T= 2,45; α = 5%), indicating an acidification process in function of the introduction of excrements, probably rich in uric acid (21). Besides this, the microbial decomposition of great quantities of organic matter causes the formation of organic acids, that are dissolved in the soil matrix water (3). Low pH values may be originated by the loss of bases by leaching, production of organic acids, carbonic acid dissolution, climatic conditions and rainfalls (10).

4. *Moisture content of the soil* (%) - In the affected soil the moisture content was higher in January, corresponding 69,1 %, whereas the lowest values were found in February, corresponding to 58,97 % (Table 1). In the control sites, the moisture content was higher in December, corresponding to 84,18 % and lower in March, corresponding to 68,91 %. The mean values of the soil moisture were statistically lower in the affected soil in November (T= 4,38; α = 1 and 5%), December (T= 6,16; α = 1 and 5%), January (T= 4,61; α = 1 and 5%), February (T= 5,39; α = 1 and 5%) and March (T= 2,62;α = 5%).

The drying effect of the soil can be a consequence of a hygroscopic characteristic of the organic substances contained in the excrements of the birds (S. M. Tauk-Tornisiello, personal communication) and the parcial destruction of the vegetation resulting in a greater exposition of the soil to climatic conditions.

RESUMO

Fungos isolados no solo afetado pela presença de aves no "Parque Estadual das Fontes do Ipiranga", São Paulo, Brasil.

Com o objetivo de verificar possíveis efeitos acarretados por dejetos de aves sobre a micota e algumas características abióticas do solo situado às margens do Lago das Garças no Parque Estadual das Fontes do Ipiranga, SP, foram coletadas mensalmente, de outubro de 1990 a março de 1991, amostras de solo de cinco áreas afetadas pela presença das aves e outras cinco amostras de áreas consideradas controle. Os fungos foram isolados por meio da técnica de placa de solo consorciada à técnica de iscagem com substratos celulósicos, quitínicos e queratínicos. Foram isolados no total 30 táxons de fungos, sendo 22 Deuteromycotina (Hyphomycetes), 01 Ascomycotina (Plectomycetes), 07 Mastigomycotina (03 Oomycetes e 04 Chytridiomycetes). As médias de temperatura do ar e do solo a 15cm de profundidade não foram significativamente diferentes nas áreas estudadas. O pH e o teor de umidade apresentaram valores significativamente menores no solo afetado pelas aves em relação ao solo da área de controle, possivelmente decorrente do efeito hi-

groscópico e ácido de excrementos lançados ao solo. As médias do número de colônias de fungos determinadas mensalmente apresentaram-se significativamente maiores no solo afetado pelas aves, ao passo que a diversidade das espécies foi menor, indicando possíveis restrições do ambiente sob impacto sobre os fungos nativos do solo.

Palavras-chaves: aves, fungos do solo, lago.

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CADMIUM UPTAKE AND ITS EFFECT ON THE GROWTH OF *CHLORELLA HOMOSPHAERA* AND *SCENEDESMUS QUADRICAUDA* CELLS IN LABORATORY CONDITIONS

Selma G. F. Leite[1]
Antonio Carlos A. Costa[2]
Marcelo C. Moore[1]
Gustavo A. S. Pinto[1]

ABSTRACT

The toxic effect and uptake of cadmium during growth of *Chlorella homosphaera* and *Scenedesmus quadricauda* cells was investigated at different initial cell concentrations (0.1 to 2.0 g/l). The cells were grown in synthetic growth medium containing added cadmium chloride to final concentrations ranging from 0.0 to 12.0 mg/l. The growth of *Chlorella* and *Scenedesmus* cells was affected by 4.0 mg/l and 2.0 mg/l of the metal, respectively. For both species, this effect was directly proportional to the metallic ion concentration and inversely proportional to the cell concentration.

Key words: cadmium uptake, green microalgae.

INTRODUCTION

The toxic effect of heavy metals on microbial cells is being investigated by various researchers. Metal accumulation promotes several changes in cells, such as alterations in cellular growth (1), cellular division and photosynthetic activity (11). Different microorganisms exhibit mechanisms for the uptake and accumulation of metals, including bacteria (13), fungi (14), marine algae (5, 8), or green microalgae (10).

The interactions microbe-metals are dependent on the source and concentrations of the metallic ion, microbial species involved and physiological and environmental aspects. Previous research indicated that many microorganisms are able to grow in relatively high concentrations of toxic heavy metals by means of different detoxification mechanisms.

This ability is attractive in the recovery of metals (7), or to reduce environmental damage. Several green microalgae, are being described in the literature as potential organisms for the uptake of metals, specially in aquatic environments.

Microorganisms from the genus *Chlorella* and *Scenedesmus* are unicellular, nutritionally not complex, their sizes ranging from 1.5 to 5.7 µm diameter. Their sources of nitrogen are basically nitrate and urea, being their nitrogen content between 7 to 11%. They are planctonic algae, usually found in freshwaters.

Cells from the genus *Chlorella* contains autospores, formed by divisions inside the cells, and chloroplasts showing a narrow opening named "mantel-shaped". Cells from the genus *Scenedesmus* have their total number of divisions limited by a cell envoltory, previously fixed.

1. Escola de Química, Depto. de Engenharia Bioquímica, Bloco E, Universidade Federal do Rio de Janeiro.
2. Centro de Tecnologia Mineral, CETEM/CNPq, Rua 4, Quadra D, Cidade Universitária, RJ, Brasil. (Corresponding author) 21949 - Rio de Janeiro - R.J.

The aim of the present study was to examine the growth and uptake of cadmium by *Chlorella homosphaera* and *Scenedesmus* quadricauda at different cadmium ion concentrations, as a study on the employment of these species for the treatment and control of metallurgical effluents.

MATERIALS AND METHODS

Organisms: Green microalgae *Chlorella homosphaera* and *Scenedesmus quadricauda*, isolated from Quinta da Boa Vista Lake (Rio de Janeiro City). The organisms were grown in synthetic medium with the following composition (g/l): $NaNO_3$, 1.00; KH_2PO_4, 0.25; $MgSO_4$, 0.50; NH_4Cl, 0.05; $CaCl_2$, (0.05); $FeCl_2$, 0.008 and glucose, 10.00. The medium was adjusted to pH 7.0. Culture medium was sterilized at 0.5 atm for 20 minutes.

Experimental procedures: The experiments were conducted in Erlenmeyer flasks containing 200 ml of medium, incubated in a rotary shaker Model Arthur Thomas, at 150 rpm at 30°C for 100 hours under fluorescent illumination with 13000 lux of intensity. Each experiment was conducted four times. They were carried out at different cell concentrations: 0.1, 0.5, 1.0 and 2.0 g/l. Cadmium chloride, was added to the growth medium in concentrations ranging from 0.0 to 12.0 mg/l.

Cadmium chloride solutions were prepared from a concentrated stock solution, autoclaved at 111°C for 15 minutes and diluted for use in the experiments.

Quantifications: Cell growth was measured turbidimetrically at 430nm, and related to algae dry weight through a calibration curve. Cadmium uptake was determined in the supernatant, after cell centrifugation at 200 rpm for 15 minutes followed by filtration in 0.47 µm Millipore membranes, by atomic absorption spectrophotometry using Varian Techtron Spectrophotometer, Model AA6. The spectrophotometer was previously calibrated with a series of cadmium solutions prepared from a concentrated Merck standard cadmium solution.

RESULTS AND DISCUSSION

It was found that cellular growth ceased after 45 hours in cadmium free medium, in all tested conditions, the highest cell concentration obtained being 4.0 g/l, for *Chlorella* cells (Figure 1). The same final biomass was reached more quickly when the initial inoculum was increased. A 2.0 mg/l cadmium concentration did not affect cell growth of *Chlorella* significantly. These results are not in agreement with the literature (1,12), where the inhibition of growth for *Chlorella* cells was reported at cadmium concentrations lower than 2.0 mg/l. Our report shows negligible effect on cell growth at this cadmium concentration, but with increasing metal concentration the growth rate is reduced; this way, only a 2.0 g/l initial inoculum achieves maximum cell growth in presence of 12.0 mg/l. These observed profiles follow certain information in the literature about cadmium toxicity on algal cells and its effect on cell growth. Otherwise, the results were surprising due to the high metal concentrations employed, partially affecting the growth of *Chlorella homosphaera* cells. This is probably associated to the use of a culture medium providing optimum conditions for cell growth. The absorption curves for different cadmium concentrations are not similar. It appears that the amount of cadmium absorbed by a specific cell inoculum diminishes as the metal concentration increases. The absorption becomes less effective as a function of cell contamination due to the presence of the metal, although it is complete at the final stages of the process. It is very interesting to notice the instantaneous metal uptake by the cells, a fact already detected by us and other researchers (2, 6, 10).

The growth of *Scenedesmus quadricauda* in cadmium containing medium was inhibited in all metals concentrations tested (Figure 2). The cells were again more resistant to ions effect in the highest inoculum, but still showed the repressive effect of cadmium on the final biomass growth, at the test conditions.

Doyle et al. (3) studying bacteria and molds reported a negative influence in almost all microorganisms in the presence of cadmium at 5 µg/l. However, the growth of *Escherichia coli* and *Streptococcus faecalis* was stimulated with 5 and 10 µg/l of cadmium, respectively.

In parallel we could observe cadmium uptake from solution. This was complete up to 6.0 mg/l as a initial metal concentration, even with a decrease in cell growth. The time required for metal accumulation increases with the metallic content of the medium. This fact can be understood if the metallic ion does not promote total inhibition of the metabolic activity. In this case cell growth is obtained. On the other hand it will generate more binding sites for cadmium uptake, indicating that algal cell walls (4) and extracellular material (9), are efficient biosorptive agents. On the other

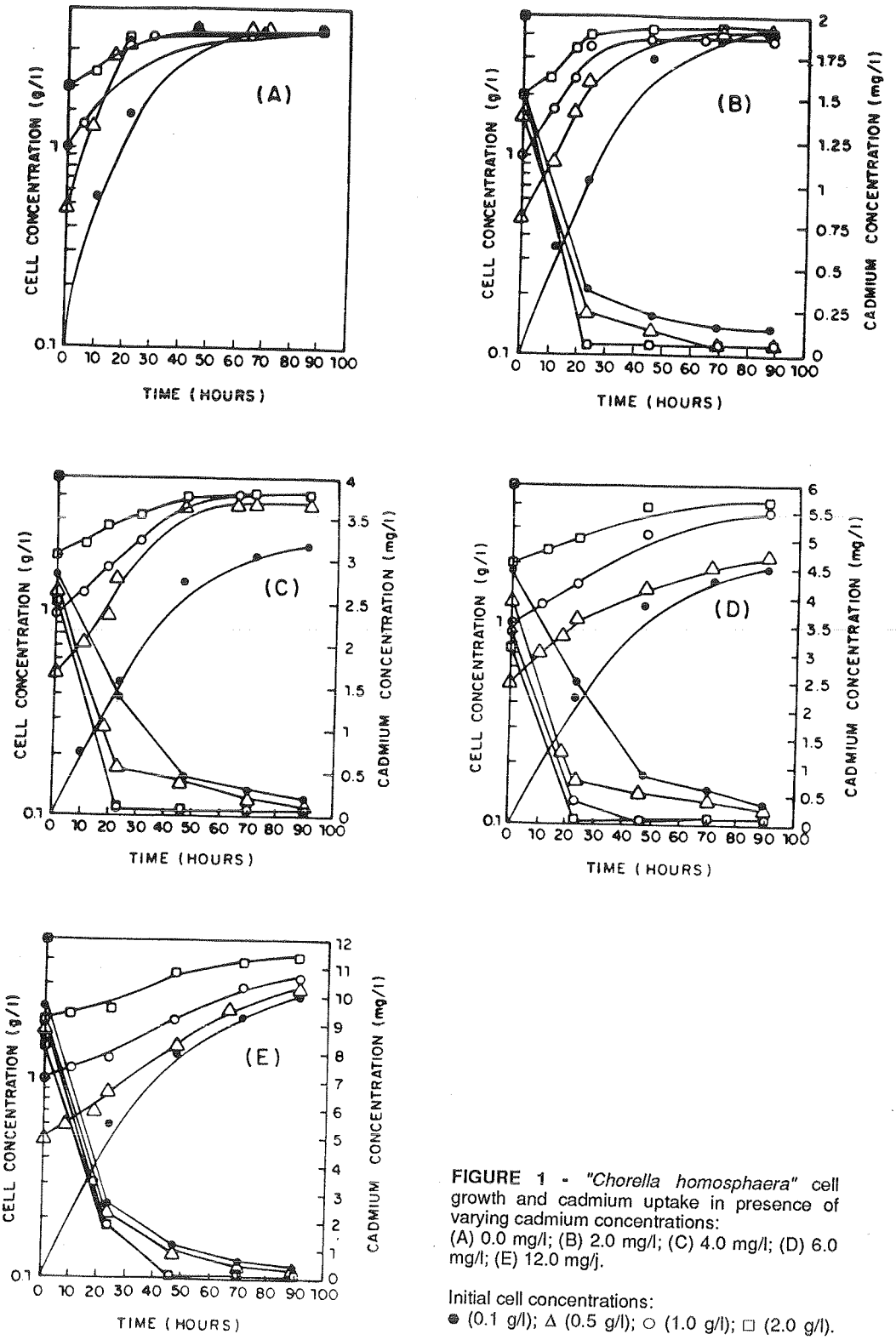

FIGURE 1 - *"Chorella homosphaera"* cell growth and cadmium uptake in presence of varying cadmium concentrations:
(A) 0.0 mg/l; (B) 2.0 mg/l; (C) 4.0 mg/l; (D) 6.0 mg/l; (E) 12.0 mg/j.

Initial cell concentrations:
● (0.1 g/l); Δ (0.5 g/l); ○ (1.0 g/l); □ (2.0 g/l).

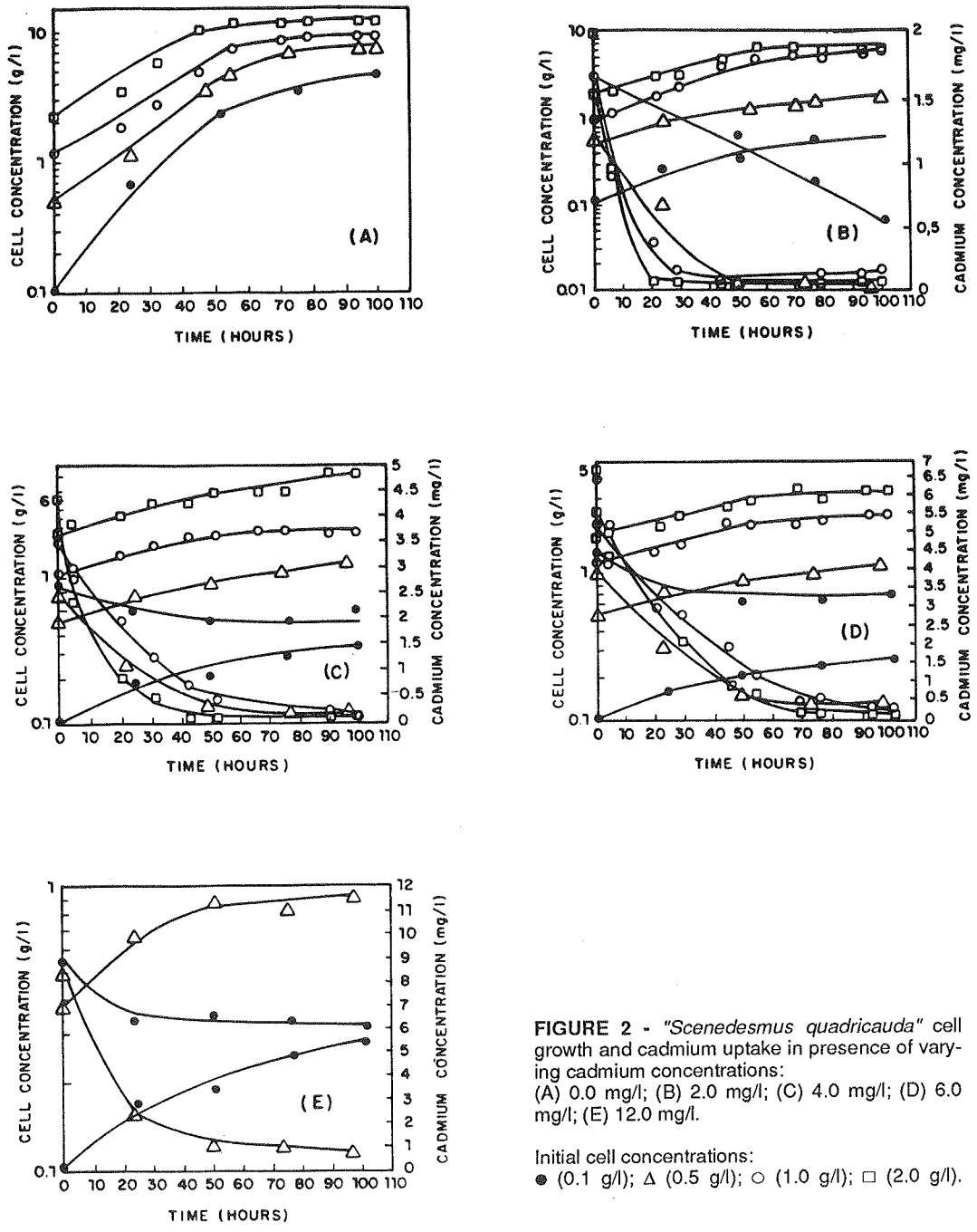

FIGURE 2 - *"Scenedesmus quadricauda"* cell growth and cadmium uptake in presence of varying cadmium concentrations:
(A) 0.0 mg/l; (B) 2.0 mg/l; (C) 4.0 mg/l; (D) 6.0 mg/l; (E) 12.0 mg/l.

Initial cell concentrations:
● (0.1 g/l); Δ (0.5 g/l); ○ (1.0 g/l); □ (2.0 g/l).

hand, the *Scenedesmus quadricauda* strain has shown to be extremely sensitive to cadmium in this purpose.

Experiments carried out in this way provide a favourable indication of the potential applicability of the sorption process for cadmium containing industrial effluents clean-up by *Chlorella homosphaera* cells. These results were obtained for a preliminary evaluation of the feasability of using green microalgae as metal sorbents. After being captured, the metals, concentrated in the cell components, can be efficiently eluted. This is an important feature both industrial clean-up and for the concentration of heavy metals.

RESUMO

Captação do cádmio e seu efeito no crescimento de *Chlorella homosphaera* e *Scenedesmus quadricauda* em condições laboratoriais.

O efeito tóxico e a captação de cádmio durante o crescimento de *Chlorella homosphaera* e *Scenedesmus quadricauda* foram estudados em diferentes concentrações iniciais de células (0,1 a 2,0 g/l). As células foram crescidas em meio de crescimento contendo cloreto de cádmio nas concentrações de 0,0 a 12,0 mg/l. O crescimento celular de *Chlorella* e *Scenedesmus* foi afetado pelo metal nas concentrações de 4,0 mg/l e 2,0 mg/l, respectivamente. Para as duas espécies estudadas, este efeito foi diretamente proporcional à concentração do metal e, inversamente proporcional à concentração celular.

Palavras-chaves: Absorção de cádmio, microalgas verdes.

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STUDY OF AMNONIFYING BACTERIA BEHAVIOR IN THE LAKES CARIOCA AND D. HELVECIO (RIO DOCE VALLEY - MG)

Pedro Magalhães Lacava[1]
Célio Augusto Rugani[2]

SUMMARY

The study of the amnonifying bacteria behavior in the lakes Carioca and D. Helvécio, which belong to the natural lacustrine system of the Rio Doce Valley - Minas Gerais - Brazil, during the period from March to November, showed a similar seasonal variation in both lakes, with higher population density in March and November (raining period) and lower density in May, July and September (dry period). The vertical variation was also relatively similar for both lakes, were during the thermal stratification the distribution was regular along the water column, while during the period of thermal stratification the populational density was remarkably different at different depths. The characterized ammonifying types to the genera *Acinetobacter, Moraxella* and *Proteus,* which in culture reveal high amnonifying activity with values which have reached 30,8 µg/l of ammonia per population unity (105 bacteria/ml).

Key words: ammonifying bacteria, aquatic bacteriology, *Proteus, Moraxella, Acinetobacter.*

INTRODUCTION

The role of the amnonifying bacteria in the process of decomposition and mineralization of organic material and, therefore, supply of nitrogen nutrients to primary products is of fundamental importance. According to other authors (10, 15, 17) nitrogen combined do NH^3 and NO_3^- is considered as one of the principal macronutrients which lead the primary production. This process result, furthermore, on biosynthesis of particulated protein established by the bacterial cells supplying excelent food to the zooplacton (2, 8).

In that manner, this study have aim to isolate and characteraze amnonifying bacteria in two natural lacustrine surroundings (lakes Carioca and D. Helvécio belonging to the Rio Doce Valley System of lakes - MG) described by De Meis (6), and also to delimit the seasonal cicle and the quantitative distribution of this amnonifying bacteria in samples taken from vertical profiles, besides avaliate it's amnonifying capacity in pure cultures.

MATERIAL AND METHODS

1. Colleching samples - stablished a collect sation in the central part of each lake, the amnonifying bacteria population's raising has been made bimonthly, starting in March and ending in November. The months of March and November con-

1. Instituto de Química - UNESP - Caixa Postal 355 - 14800 -900 Araraquara - SP.
2. Instituto De BiociÊncias - UNESP, Caixa Postal 178 - 13500 - Rio Claro - SP - BRASIL.

stitute the rain season and the dry season is represented by the months of May, July and September.

Samples were collected on penetration profundity of the green light (500 - 650nm) at 100, 50, 25, 10, 1% and on aphotic zone, through a Van Dorn bottle, previously washed with the same water of the collecting point and fartherly transfered to sterilized receptacles (600 ml) and conducted immediatly to the laboratory.

2. Estimative, isolation and characterization of amnonifying bacteria - the culture media used to estimate amnonifying bacteria presence was nutrient agar (Difco) added of red of cresol in the proportion of 1/50 of a solution 0,1%. Using three volumes of different innocula: 0,1; 0,05 and 0,01 ml, employing the tecnic of scoring viable cells by scattering with a loop of Drigalsky. The temperature of incubation stayed around 27°C (±3°C) with scoring done after 24, 48, 72, 96 and 120 hours of incubation, being considered the largest reading attained. The amnonifying change the indicator promoting the formation of a red halo around the colony contrasting to the rest of the plate which is yellow.

The representative colonies were isolated, purified and stocked in nutrient agar. To identification were made biochemical proofs and tests according to BUCHANAN and GIBBON (3) abd SKERMAN (13) besides the macroscopic observations to morphology of the colonies and microscopics for the cells forms.

The biochemical proofs and tests executed were as follous: oxidase production, catalase production, growth in culture medium containing KCN, production of acid from glucose, production of lysine, arginine and glutamic acid dehydrogenase, production of acetil-metil carbinol, utilization of sugares (arabinose, sucrose, dulcitol, glucose, lactose and manitol). Utilization of citrato, productions of indol, hydrogen sulfid, urease and ammonia. As soprophitic bacteria have vast physiologic diversification it is very difficult to have accurately identification and classification throughout biochemical tests. The overcome of this difficulty was done through the use of numerical toxonomy consisting the determination of indexes of similarity to each poir of bacteria groups (13).

3. Avaliation of ammonia production - the isolated heterotrophic bacteria were primary submited to ammonia production tests through Nesler and Krup indications (12). After the selection of amnonifyings, these were innoculated in nutrient broth (Difco) and incubated for 48 hours at 30°C, intending to obtain young and growth exponential fases cells. Aliquots of 1 ml have been withdrawn from each one cultures and innoculated in six tubes with nutritive broth, closed with rubber stoppers to avoid the losing of the produced ammonia. Concomitantly to this innoculation in tubes, were made alse a plating after seriated dilutions in nutrient agar to scoring the number of bacteria in the culture. Tubes and plates were incubated at 30°C. The dosage of produced ammonia by the various bacterial types were determinated by titulometry, according to Golterman (7) after two, five and seven days of incubation.

RESULTS AND DISCUTION

1. Amnonifying Bacteria: Vertical distribution and seasonal variation - Table 1 express bacterial quantities vertically distributed in different profundities during the period of March and November. Vertical distribution is determinated mainly by Thermic stratification and destratification of the lakes. On May and July when there were circulation of water mass (destratification) the bacterial population has distributed itself more homogeneously and in minor density in the whole water line and were relatively similar in the two lakes. Similar results were found in other lakes (1) (4).

Table 2 and figure 1 show the season variation in the refered period with values expressed as meddle of the total of cells obtained on all water line. The results show the seasonal variation quite defined and similar to both lakes, with more occurrence of amnonifyings on summer months, March and November (raining period) and minor population density in May, July and September during dry period. The pluviometric precipitation and the temperature are the factores which emerge as the most probable accountable for seasonal variation (1, 16, 9, 5, 11).

2. Isolation and Identification of amnonifying bacteria - Isolated colonies were of seven (7) bacterial types of amnonifyings. After the gothering of this types in similarity inceasing order, was possible to set them in three different genera: *Acinetobacter, Moraxella e Proteus.* In table 3 the morpho-physiologic characters of bacteria are recorded.

3. Estimative of ammonia production by isolated bacteria - Table 4 and figure 2 show results obtained on ammonia production in culture media by bacterial types. Expressed results reved that this bacteria determinate a high production of ammonia. Within limits of inicial population units

TABLE 1 - Vertical distribuition of amnonifyingheterotrophic bacteria (x10^2 / ml) on correspondents profundities at 100, 50, 25, 10 and 1% of (green) light penetration and aphotic zone, at Carioca and D. Helvécio Lakes, during the period of March to november.

Carioca		D. Helvécio	
PROF. (m)	BACT (x10^2 /ml	PROF. (M)	BACT (x10^2 /ml
	March		
0,0	143,0	0,0	95,0
0,7	20,0	1,5	12,0
2,7	22,0	3,0	2,2
4,0	17,0	6,0	3,2
6,5	53,0	14,0	6,2
-	-	18,0	46,4
	May		
0,0	3,0	0,0	0,8
0,4	4,0	1,7	11,6
1,0	3,6	3,0	6,3
1,5	3,6	6,0	3,4
4,0	3,2	14,0	3,5
9,0	6,0	20,0	2,2
	July		
0,0	4,2	0,0	6,8
0,7	7,0	1,2	5,2
1,3	4,6	4,0	1,5
2,9	3,8	8,5	1,4
6,0	2,1	14,5	1,2
8,0	4,5	18,0	1,8
	September		
0,0	3,1	0,0	1,8
0,6	7,0	1,7	24,6
1,2	6,6	4,0	6,8
1,9	7,0	8,5	9,4
4,5	3,4	16,5	24,8
9,0	6,6	20,0	6,4
	November		
0,0	15,0	0,0	1,6
0,6	7,0	1,0	4,0
1,1	7,0	3,0	12,8
2,2	5,8	7,3	3,4
5,0	22,0	15,0	78,2
8,0	8,0	20,0	2,0

(10^5 bacterial ml) the type 6 (genus Proteus) has shown a production of 30,8 30,8 µg/l of ammonia considerated the greatest productor, leaving to the type 7 (genus moraxella) the minor production with 0,01 µg/l of ammonia. In aquatic surroundings there is a significant relation between ammonia production and number of amnonifying bacteria present (14) (9).

TABLE 2 - Seasonal variation of amnonifying bacteria (bacteria average number x 102 /ml) in Carioca and Helvécio Lakes, at the period of March to Novemver.

Month	Carioca	D. Helvécio
March	51,0	23,8
May	3,9	4,8
July	4,4	3,0
September	5,6	12,3
November	10,8	17,0

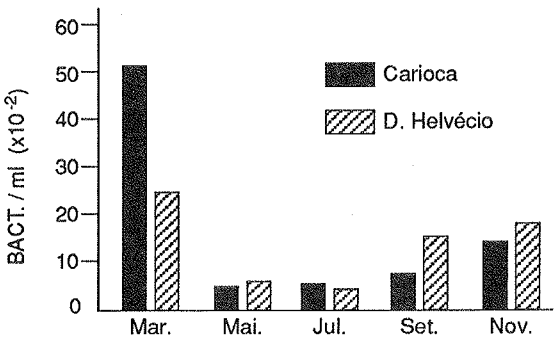

FIGURE 1 - Seasonal variation of amnonifying bacteria (Average rote on water column x 10^{-2} / ml), on Carioca and D. Helvécio Lakes.

TABLE 3 - Morphological and physiological characteristics of the isolated amnonifying bacteria on Carioca and D. Helvécio Lakes.

Genus	Proteus	Moraxella	Acinetobacter
Type	1 4 6	2 5 7	8
Gram	- - -	- - -	-
Motility	+ + +	- - -	-
Colony	cr am cr	cr cr cr	am
Sporous	- - -	- - -	-
Catalase	+ + +	+ + +	+
Oxidase	- - -	+ + +	-
Kcn	+ + +	+ + +	+
Glucose / Gas	- - -	- - -	-
Arabinose	- - -	- - -	-
Dulcitol	- - -	- - -	-
Glucose	+ + +	- - -	+
Lactose	+ + +	- - 2 -	-
Manitol	+ + +	- - -	-
Sacarose	+ + +	- - -	-
Glicose / Ácido	- + -	- - -	-
Voges-p=Proskauer	+ + +	+ + +	+
Citrato	- - +	+ + -	-
Glutamic Acid	+ + -	- - -	-
Arginine	- - -	- - -	-
Lysine	+ - -	- - -	-
H_2S	- - -	+ + -	-
Indol	- - -	- - -	-
NH_3	+ + +	+ + +	+
Urease	+ + +	- - -	-

cr = Cream
am = Yellow

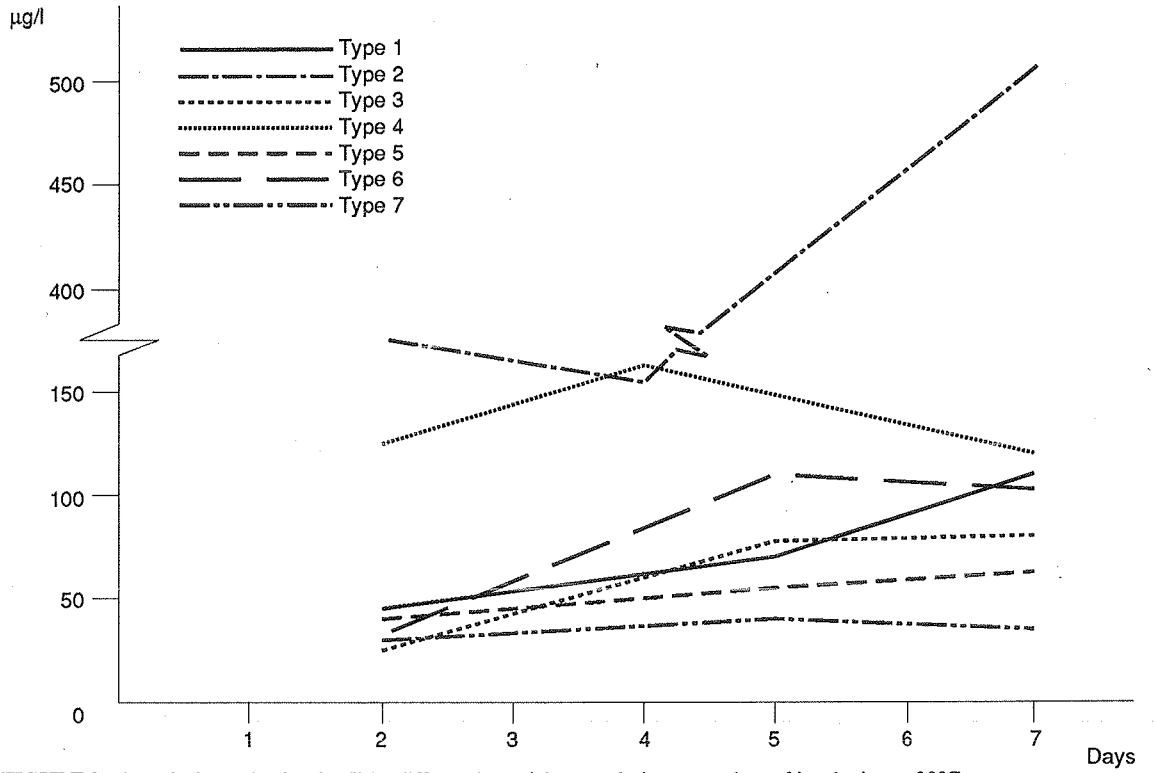

FIGURE 2 - Ammômia production (µg/l) by different bacterial types, during seven days of incubation at 30°C.

TABLE 4 - Ammonia production (µg/1) by different Bacterial types, during seven days of incubation at 30°C.

Type	Start Cells	Ammonia (µg/1)		
Bact	x 105 / ml	2 days	5 days	7 days
1	50	42,9	75,9	114,4
2	2200	169,4	145,2	497,2
4	34	27,5	80,3	84,7
5	2300	132,0	149,6	123,2
6	2	37,4	57,2	61,6
7	6800	35,7	115,5	112,2
8	19	30,8	37,4	30,8

RESUMO

Estudo do comportamento de bactérias amonificantes nos lagos Carioca e D. Helvécio (Vale do Rio Doce - MG).

O estudo do comportamento de bactérias amonificantes nos lagos Carioca e D. Helvécio, pertencentes ao Sistema lacustre natural do Vale do Rio Doce - Minas Gerais - Brasil, durante o período de março a novembro, demonstrou uma variação sazonal similar nos dois lagos, com maior densidade populacional em março e novembro (período chuvoso) e menor densidade em maio, julho e setembro (período seco). A variação vertical também foi relativamente similar para ambos, onde durante a desestratificação térmica a distribuição foi regular em toda a coluna d'água, enquanto que durante o período de estratificação térmica a densidade populacional foi marcadamente diferente nas várias profundidades. Os tipos amonificantes caracterizados pertencem aos gêneros *Acinetobacter, Moraxella* e *Proteus*, revelando em cultura uma alta atividade amonificante com valores que atingiram 30,8 µg/l de amônia por unidade populacional (10^5 bactérias/ml).

Palavras-chave: Bactérias amonificantes, bacteriologia aquática, *Proteus, Moraxella, Acinetobacter*.

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GROWTH BEHAVIOR OF *CHLORELLA* SPECIES IN AUTOTROPHIC AND MIXOTROPHIC ENVIRONMENTS - A COMPARATIVE STUDY

Márcia Teresa S. Lutterbach
Paschoal Guimarães Robbs[1]

INTRODUCTION

The world supply of proteins, vitamins and minerals can not be accomplished by the usual production means (7). Therefore, there is a need to investigate alternative sources of foods and animal feeds (2).

There are many ways for utilization of microalgae, such as (a) in human foods, specially as supplementation of protein and vitamins; (b) in animal feed (16,18); and (c) in waste water treatments (8).

Human consumption of microalgae has been a commom practice for many centuries (5). Researchers from many parts of world have been studying microalgae as a non-conventional protein source (2,14) due to the good aminoacid profile and the low nucleic acid concentration when compared to yeasts and bacterias (8).

Growth and composition of *Chlorella* sp. are affected by many variables, most of them interrelated. Changes in chemical composition and morphology may be a function of environmental conditions (20), such as the nitrogen source used (12).

The utilization of microalgae is increasing in mixotrophic growth, mainly for waste water treatments. Therefore, it can be very important to know the strain capability to absorb organic materials which is a physiologic characteristic that vary among the species (19).

Adequate climate for algae growth is encountered in many parts of Brazil. Temperature and solar radiation, essential factors for appropriate algae development, are even better in Brazil than in many other producing countries. In addition, the alcohol program generates CO_2 as by product, usually discharged to the atmosphere, which could be used as carbon source by the algae (6).

Potencial of microalgae (18), their nutritional value according to the modifications in growth media (1, 16, 18, 20), applications as animal feed and utilization in sewage purifications (8) are some of the studies being conducted to verify uses of microalgae in Brazil.

The Department of Food Technology at the University Federal Rural do Rio de Janeiro has been developing a research program to increase the knowledge in the area of algae growth, biomass production (SCP), as well as the extraction of compounds and food fortification. This research is part of such project and has the following objectives: to study the growth behavoir of four strains of *Chlorella* in different culture media and nitrogen sources; to evaluate the algae growth using organic carbon materials, and to verify possible morphological variations under some environmental conditions.

MATERIAL AND METHODS

Microorganisms - Four strains of *Chlorella* were used in the experiments: *Chlorella vulgaris* var. *viridis* strain Utex 30 (ATCC 16 487), *Chlorella vulgaris* strain Cu 211/11H (ATCC 11 468), *Chlorella ellipsoidea* strain Pringshein (ATCC 11 466) and *Chlorella homosfera* (UFRJ).

All the algae were maintained in mineral modified Allen medium containing 1,5% of Agar-Agar, at 28 to 30°C and under fluorescent lamp (GE 20 watts). Upon growth completion, the samples were kept in a refrigerator (4 - 7°C). Repication was done every three months.

Culture media - Allen, 1952 - without micronutrients, as modified by Araújo, 1976 (1), Pratt, 1963 (15) and Sorokin & Krauss, 1958 (21) were the media used, as recommended for green microalgae growth.

Departamento de Tecnologia de Alimentos, Instituto de Tecnologia, UFRRJ. Km 47 Antiga Rio-São Paulo - Itaguaí (CEP 23851) - Rio de Janeiro - RJ - Brasil.
1. CNPq researcher

Growth control - Cell growth was evaluated by measurement of optical density (O.D.) was taken at 430 nm in Bausch & Lomb spectrophotometer (Spectronic model 20) to evaluate cell growth. Dry weight (D.W.) (mg/100ml) was determined after cell washing and drying at 105°C. The relationship D.W./O.D. was also calculated.

Cell size was measured by a binocular microscope (Carls Zeiss Jena) equipped with a 10 fold increase graduated ocular. Fifty cells from each growth medium were measured according to the technique described by Martelli & Rosemberg (13). The mean and standard desviation were calculed.

The pH was determined in a Cg 711-Schottmainz pH meter.

The method mentioned by De Stefanis and Ponte Jr. (4) was employed to verify the pureness of the sugars utilized on the experiments.

Purity of the algae cultures was regularly verified by direct microscopic observations.

Growth conditions and experiment control - Evaluation of autotrophic growth - The procedure to obtain the inoculum consisted in preparing a cell suspension in a 5 ml sterile buffer solution using a stock culture. The suspension was them placed into four 1000 ml Erlenmeyers containing 200 ml of modified Allen medium solidified with 1.5% Agar-Agar. After incubation under constant light exposure during 5 to 7 days, a suspension was obtained and further used as inoculum in a proportion of 100 mg of cells/100 ml of culture medium.

The growth of algae strains were observed in the three media described previously. The experiments were carried out in Erlenmeyers containing 100 ml of a specific medium. Uniform light flux was provided by 4 fluorescent 20 watts lamps over the Erlenmeyers, and kept at 30 cm from the culture surface. The temperature was mantained at 30°C +/- 1°C using a water-bath-shaker (Dubnoff FANEN model 144) at 120 RPM. CO_2 level was that available from the atmosphere. Samples were drawn each 24 hours to determine pH, $O.D._{430}$ and cell size. Each experiment was run for 120 hours.

Nitrogen source assay - The inoculum was prepared as previously described. Three different nitrogen sources (NH_4Cl, Merck; $Na\ NO_3$, Merck; and urea, Reagen) were added to the modified Allen medium without nitrogen. The nitrogen concentration of the new sources was maintained at the same level as the original content (0.295 g/l).

The algae growth were performed in 100 ml of each growth medium, using 500 ml Erlenmeyers. All variables were kept at the same control level, as described in the section of growth conditions and experiments control; however, the growth time was reduced to 72 hours.

Carbon source assay - The following sterile carbon sources were added into the sterilized modified Allen medium (45 ml of medium in 250 ml Erlenmeyers) to a final carbon concentration of 1%: acetic acid (Mast), ethyl acetate (Carlo Erba), dextrose (Vetec), ethyl alcohol (P. A. grade), fructose (Eastman Kodak), galactose (Merck), lactose (Merck), maltose (Carlo Erba), sucrose (Hoescht), xylose (Merk). Adjustment of pH to 5.5 +/- 0.1 was performed by additions of sterile solution of NaOH or H_2SO_4.

The experiments were conducted as mentioned previously during 120 hours. Cell size was determined at time zero and after 120 hours. Dry weight was also calculated afetr 120 hours.

RESULTS AND DISCUSSION

Biomass production differed according to the medium (figures 1 to 4). It rangeg from 0.17 g/l for *C. ellipsoidea* to 0.53 g/l for *C. vulgaris*, after autotrophic growth of 5 days. Such slow growth rate was expected, since only atmospheric CO_2 was available, what could have been a limiting factor. There are reported in the literature similar results (18). Addition of extra CO_2 improved significantly cell production in some studies (16). However, the algae growth under atmospheric pressure of CO_2 provides important information concerning the capability of absorption of CO_2 under low dissolved gas concentration, photosyntesis efficiency and general metabolism. These conditions have also been used in other investigations (1,16,18).

C. vulgaris (figure 1), showed higher growth rate and yield in modified Allen medium. The other strains, *C. homosfera* (figure 2), *C. ellipsoidea* (figure 3) and *C. vulgaris* var. *viridis* (figure 4) presented similar results in all the three media. Araújo (1) also could not detect any difference in the growth rate when using modified Allen and Pratt media to obtain *C. homosfera* cells.

The buffering capacity, an important characteristic of mineral media used to grow microalgae, was higher for Pratt medium due to the presence of p65 xYhates in its composition. It can be observed in figures 1 to 4, that the pH remained stable in the range from 5.2 to 6.0. However, detectable variation was observed in the alkaline re-

gion, when using the Sorokin & Krauss medium (figures 1 and 2). Modified Allen medium showed the worst buffering power. All the species of microalgae tested presented higher growth rate in modified Allen medium when compared to the other two ones.

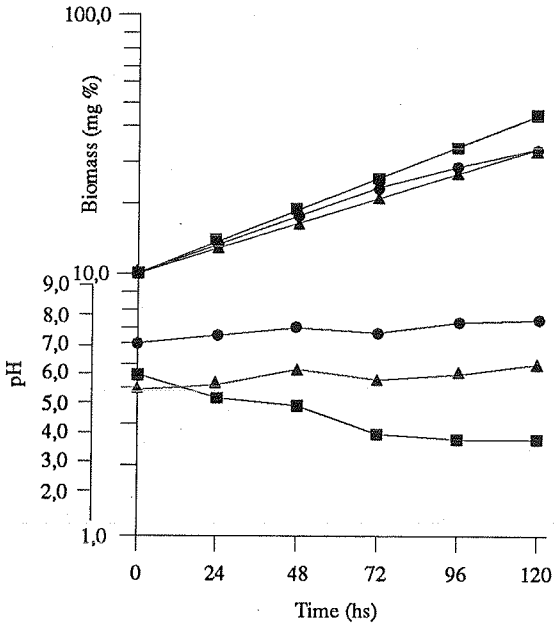

FIGURE 1 - Growth curves and pH variations for *C. vulgaris* cultivated in modified Allen —■—; Sorokin & Krauss —●— and Pratt —▲— medium.

C.vulgaris and *C. homosfera* provided the best biomass yield in all media, followed by *C. vulgaris* var. *viridis*. *C. ellipsoidea* did not grow appreciably when compared to the other two algae.

The results show insignificant difference in alga mass obtention among the three media. For this reason, the modified Allen was used to conduct the rest of this study. Besides, modified Allen had a simple composition, it could be used to compare the results obtained by other authors and changes in pH would be observed during growth.

The nitrogen content in the modified Allen was replaced by equal quantities of NO_3^-, Urea and NH_4^+, to test the effect of nitrogen sources in alga development.

The use of different nitrogen sources did not affect the growth pattern of any algae. *C. vulgaris* produced 0.22 to 0.26 g/l, *C. homosfera* 0.20 to 0.23 g/l, *C. vulgaris* var. *viridis* 0.17 to 0.19 g/l and *C. ellipsoidea* 0.09 to 0.13 g/l of biomass during 72 hours. Addition of NH_4^+ caused a pH

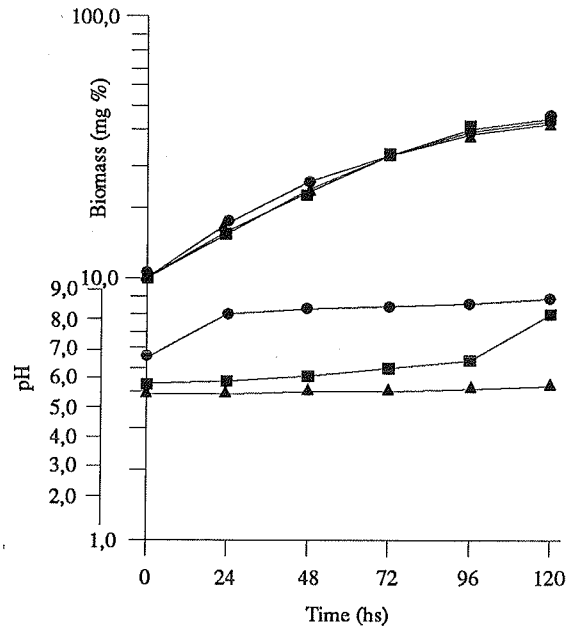

FIGURE 2 - Growth curves and pH variations for *C. homosfera* cultivated in modified Allen —■—; Sorokin & Krauss —●— and Pratt —▲— medium.

decrease, a fact also noticed by other researchers (5,11,16,19). NO_3^- added to the medium brought on a pH increase, while urea maintained the pH relatively stable. Concentration as low as 1mM of NH_4^+ can inhibit algae development (9), what might be related to the penetration of undissociated amonium hydroxide (NH_4 OH) into the cell, causing pH increase. Other effects, mainly related to the photosynthesis, are also discussed in the literature (23). However, in this research, the inhibitory action of NH_4^+ could not be detected and the use of any nitrogen compound (NO_3^-, NH_4^+ and urea) resulted in similar biomass production after growth for 72 hours. Advantages in the use of NO_3^- and urea in alga growth are reported in the literature (11,14). Theoretically, NH_4^+ would be the best nitrogen source because the cell would not need to produce enzymes for its use in amination processes; for the others compounds, enzyme production by the microorganism would be necessary (9).

C. ellipsoidea presented the worst growth rate, compared to others two algae, when nitrate was the nitrogen source. Nevertheless, higher rates were obtained using urea. These facts might indicate adaptation problems at different environmental conditions. Decreases in *C. ellipsoidea* biomass yield were always detected during the first 24 hours of

growth. *C. vulgaris* and *C. homosfera* showed similar behavior in all three media and *C. vulgaris* var. *viridis* showed a slightly lower performance.

Slight differences in cell diameter (table 1), were observed for the three mineral media used in autotrophic experiments. The smallest cell diameter mean was observed for *C. homosfera* - 3.5 +/- 0.7 µm - and the biggest for *C. ellipsoidea* - 4.5 +/ - 0.3 µm. However, these values were in accordance with the values for the *Chlorella* sp., 4 to 7 µm determined by Endo et al. (5) and 2 to 12 µm by Tyml (22).

TABLE 1 - Cell diameter (µm) variations for *Chlorella* species cultivated in modified Allen (m. Allen), Sorokin & Krauss (S & K) asnd Pratt media during 120 hour growth period.

Specie	Medium	Time (hs.)							
		0	24	48	72	96	120	X	S
C. ellipsoidea									
	m. Allen	4.4	4.0	4.2	4.1	4.1	4.2	4.2	0.2
	S & K	4.4	4.4	5.1	4.4	4.6	4.3	4.5	0.3
	Pratt	4.4	4.6	4.4	4.3	4.5	4.4	4.4	0.1
C. homosfera									
	m. Allen	3.7	5.3	3.5	3.1	2.8	2.7	3.5	1.0
	S & K	3.7	5.1	3.5	3.1	3.1	3.4	3.7	0.7
	Pratt	3.7	4.8	3.7	3.0	2.9	2.9	3.5	0.7
C. vulgaris var. *viridis*									
	m. Allen	3.7	3.6	3.8	3.9	3.9	4.1	3.8	0.2
	S & K	3.7	4.1	3.9	3.7	3.7	3.5	3.8	0.2
	Pratt	3.7	3.7	3.7	3.9	3.8	4.0	3.8	0.1
C. vulgaris									
	m. Allen	4.0	3.7	3.6	3.8	4.1	4.0	3.9	0.2
	S & K	4.0	4.2	4.1	3.9	4.2	4.4	4.1	0.2
	Pratt	4.0	3.9	3.5	3.7	3.8	4.2	3.9	0.2

Variations in cell diameter when nitrogen source was used were observed among the species but in accordance with the values previously mentioned for *Chlorella* spp. The smallest cell diameter average - 2.66 +/- 0.1 µm - was obtained for *C. homosfera* and the largest - 5.7 +/- 0.2 µm - for *C. vulgaris*. It is important to point out that differences in cell diameter during growth could not be detected for a specific sample supplememted with different nitrogen compounds. The only exception was noticed for *C. vulgaris* var. *viridis* that showed variation in cell size as a function of the nitrogen source. For *C. vulgaris* var. *viridis* when using NH_4+, the mean diameter was 5.4 +/- 0.4 µm, and for urea and NO_3^-, 4.1 +/- 0.5 µm and 3.9 +/- 0.1 µm respectively.

Results for mixotrophyc growth can be found on table 2. *C. vulgaris* var. *viridis* was the only species not increasing in cell mass or varying in cell diameter (table 3) as a response for a carbon source, in comparison to the mineral medium. *C. ellipsoidea*, which presented the worst growth in autotrophy, showed surprising development under mixotrophic conditions, mainly using fructose or glucose as carbon sources. Sucrose and galactose promoted a slight improvement in biomass production of *C. ellipsoidea*. Cell size increased from 4.4 (mineral medium) to 5.3 um and the highest biomass production was achieved using fructose. Even though other sugars were assimilated, such fact did not affect cell size.

C. homosfera was able to metabolize fructose, galactose, sucrose very well and, to a less extent maltose (table 2). An interesting finding was that increasing in sugar concentration in the medium increased the cell size, what demonstrated the alga

TABLE 2 - Dry weigth (mg%) for *chlorella* species cultivated in mineral modified Allen medium (10 mg % inoculum) with and without organic source added, after 120 hour growth period.

C source (a)	*Chlorella homosfera*			*Chlorella vulgaris*			*Chlorella vulgaris* var. *viridis*			*Chlorella ellipsoidea*		
	1. exp.	2. exp.	X	1. exp.	2. exp.	X	1. exp.	2. exp.	X	1. exp.	2. exp.	X
—	28.0	32.0	30.0	51.0	25.0	38.0	31.0	49.0	40.0	35.0	31.0	33.0
dextrose	55.5	61.0	58.3	80.0	72.0	76.0	36.0	42.0	39.0	82.0	90.0	86.0
galactose	68.0	95.0	81.5	96.0	55.0	70.5	29.0	37.0	33.0	60.0	55.0	57.5
lactose	22.0	40.0	31.0	59.0	27.0	43.0	23.0	36.0	29.5	33.0	43.0	38.0
fructose	81.5	97.0	89.3	75.0	65.0	70.0	26.0	50.0	38.0	104.0	134.0	119.0
sucrose	45.0	67.0	56.0	81.0	57.0	69.0	34.0	44.0	39.0	77.0	42.0	59.5
maltose	54.0	26.0	40.0	38.0	32.0	35.0	24.0	30.0	27.0	32.0	37.0	34.5
xylose	28.0	33.0	30.5	51.0	22.0	36.5	13.0	34.0	23.5	32.0	33.0	32.5
alcohol	27.0	30.0	28.5	59.0	35.0	47.0	28.0	46.0	37.0	22.0	22.0	22.0
acetate	30.0	39.0	34.5	28.0	23.0	25.5	11.0	28.0	19.5	24.0	43.0	33.5
acetic acid	11.0	12.0	11.5	67.0	65.0	66.0	21.0	13.0	17.0	27.0	19.0	23.0

ability to change the morphology according to sugar content (table 3). Morphological changes were also detected when studying their behavior related to the metabolism of nitrogen sources.

C. vulgaris metabolized many kinds of sugars. It was the only species to assimilate acetic

TABLE 3 - Cell diameter (μm) variations for *chlorella* species cultivated in a medium with and without addition of organic carbon sources during 120 hour period.

C source (a)	*C. homosfera*			*C. vulgaris*			*C. vulgaris* var *viridis*			*C. ellipsoidea*		
	1	2	X	1	2	X	1	2	X	1	2	X
—	3.9	3.4	3.7	8.2	7.8	8.0	6.1	6.7	6.4	4.2	4.6	4.4
dext.	6.1	6.0	6.1	6.4	7.3	6.9	6.1	6.6	6.4	4.6	4.0	4.3
fruct.	5.8	4.6	5.2	6.4	7.6	7.0	6.3	7.1	6.7	5.7	4.9	5.3
malt.	3.4	3.2	3.3	5.5	7.3	6.4	6.7	7.3	7.0	4.7	4.3	4.5
xyl.	4.9	4.4	4.7	7.3	7.5	7.4	6.7	7.5	7.1	5.9	6.4	6.2
lact.	3.2	3.9	3.6	6.7	7.2	7.0	6.1	6.9	6.5	3.9	3.8	3.9
sucr.	4.7	4.0	4.4	5.5	7.5	6.5	6.3	7.1	6.7	5.9	4.0	5.0
galac.	5.1	4.9	5.0	6.4	8.2	7.3	6.3	7.1	6.7	4.7	4.9	4.8
aceta.	4.5	4.3	4.4	6.2	7.1	6.7	6.2	8.3	7.3	5.1	3.4	4.3
alcoh.	4.2	4.0	4.1	6.1	6.4	6.3	5.8	7.5	6.7	3.9	3.4	3.7
acetic.	2.3	3.6	3.0	8.0	8.0	8.0	5.9	4.5	5.2	2.9	3.4	3.2

(a): dext. dextrose); fruct. (fructose); malt. (maltose); xyl. (xylose); lact. (lactose); secr. (sucrose); galac. (galactose); aceta. (ethyl acetate); alcoh. (ethyl alcohol) and acetic (acetic acid).

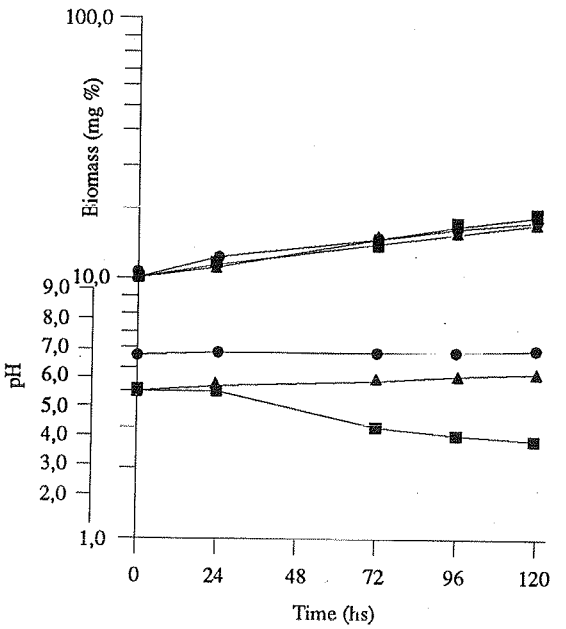

FIGURE 3 - Growth curves and pH variations for *C. ellipsidea* cultivated in modified Allen —■—; Sorokin & Krauss —●— and Pratt— ▲—medium.

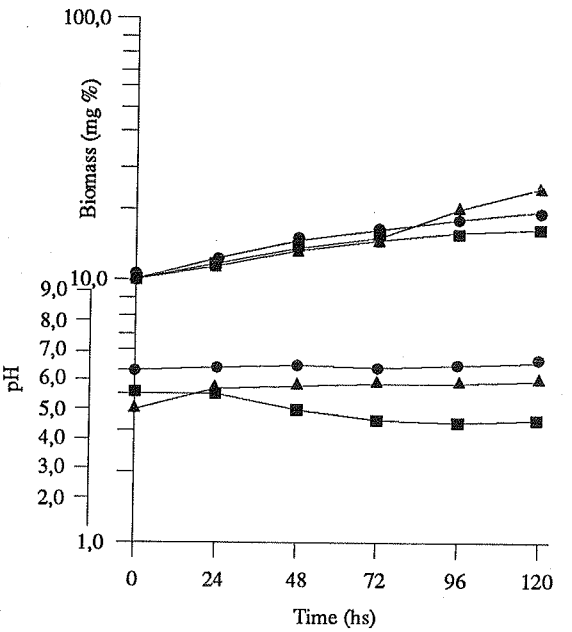

FIGURE 4 - Growth curves and pH variations for *C. vulgaris* var. *viridis* cultivated in modified Allen —■—; Sorokin & Krauss —●— and Pratt— ▲—medium.

acid and small quantities of ethyl alcohol. Satisfatory biomass weight was obtained in media containing glucose, galactose, fructose, sucrose and acetate, compared to the mineral environment. Any increase in cell diameter, as a function of the type of sugar assimilated, could not be evaluated.

Kessler (10) studied four samples of *Chlorella protothecoides*, in the dark, to verify its ability to assimilate organic carbon sources. The conclusions were: the algae were able to metabolize glucose, fructose, galactose, acetate. However, some of them grew in presence of sucrose, maltose, xylose or sorbitol. Barroso & Nonato (3), working with *Chlorella* 2L and 11L, demonstrated that the two strains used, as a nutrient, glucose and, at lower amounts, acetate but not sucrose. Glucose and galactose were considered good nutrients for *C. ellipsoidea* and *C. pyrenoidosa* growth; but sucrose was not very well assimilated (19). Rodriguez-López (17) observed that *C. pyrenoidosa*, under light and airation, significantly absorbed glucose, fructose, manose and, at lower quantities, galactose and xylose. Sucrose was not metabolized and the researcher concluded that there might exist a barrier, such as cell wall or cytoplasmatic membranes, halting the difusion of sucrose into the cell. However, the results of this re-

search indicated positive growth of all algae in media containing sucrose, except for *C. vulgaris* var. *viridis*.

The formation of giant cells with higher sugar content, in culture media having organic compounds, is reported on the literature (17). However, this fact was not observed for any strain studied in this work. It was possible to verify an increase in cell diameter. Xylose stimulated increase in diameter although not being metabolized. No explanations are available for such fact.

C. vulgaris var. *viridis* should not be recommended to treat organic residues because it did not metabolize well any organic compound investigated in this research. The other three species might have a potential application in mixotrophic environments, being *C. vulgaris* the only one able to metabolize acetate, what may be important for industrial uses.

RESUMO

Estudo comparativo do crescimento de *Chlorella* em condições autotróficas e mixotróficas.

Três meios de cultivo (o de Allen, 1952 modificado, o de Sorokin & Krauss, 1958 e o de Pratt, 1963) foram utilizados nos crescimentos autotróficos. Os cultivos foram conduzidos em agitadores recíprocos a 120 OPM, a 30 +/- 1°C por 120 hs., sob iluminação contínua, sendo o CO_2 o disponível no ar atmosférico. O melhor crescimento foi observado para *C. vulgaris* em meio de Allen modificado, tendo sido de 0,53 g/l (massa seca) a biomassa final. Esta microalga, juntamente com *C. homosfera*, apresentaram melhores resultados em todos os meios utilizados, seguida por *C. vulgaris* var. *viridis*. *C. ellipsoidea* mostrou um crescimento insignificante. As fontes de nitrogênio proporcionaram crescimentos semelhantes em autotrofia.

Variações morfológicas praticamente não foram observadas nos cultivos autotróficos, para cada espécie individualmente. Entre as espécies houve variações de 2,66 µm +/- 0,1 (*C. homosfera*) a 5,7 µm +/- 0,2 (*C. vulgaris*).

Com relação ao crescimento na presença de fontes de carbono orgânicas, *C. vulgaris*, *C. homosfera* e *C. ellipsoidea* apresentaram boas características para cultivos mixotróficos, sendo que todas responderam com aumento de biomassa a glicose, galactose, frutose e sacarose. *C. vulgaris* mostrou também a capacidade de assimilar o ácido acético, característica importante em termos industriais. Já *C. vulgaris* var. *viridis* não respondeu a nenhuma fonte.

C. homosfera foi a cepa que apresentou maiores variações morfológicas, especialmente em crescimento mixotrófico. Entretanto, células gigantes não foram formadas.

Palavras-chave: *Chlorella*, cultivo, microalgas, biomassa, tamanho celular.

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STUDY OF SOME CHARACTERISTICS OF NEWLY ISOLATED KILLER YEAST

Hélia H. Sato
Glaucia M. Pastore
Yong K. Park

SHORT COMMUNICATION

SUMMARY

Newly isolated strain of *Saccharomyces cerevisiae* 337, from sugarcane plant produced killer activity resembling the K_1 phenotype. Agarose gel electrophoresis showed that *S. cerevisiae* 337 was found to possess two dsRNA plasmids. The killer activity of the strain was compared with standard K_1 killer yeast.

Key words: Killer yeast, dsRNA plasmid, *Saccharomyces cerevisiae*.

Bevan and Makower (1) first observed that certain strains of Saccharomyces cerevisiae secreted a toxin which is lethal to other sensitive strains of the same species. The toxin is proteinaceous in nature (7). Genetic studies have shown that the killer phenotype of *S. cerevisiae* is inherited cytoplasmically and has been linked to the presence of a double stranded RNA (dsRNA) associated with virus-like particles within the cytoplasm of the killer cells (2,5). Eleven distinct patterns of the range of killer activity against killer yeast were found (K_1 - K_{11}) in accordance with the interaction between killer yeasts (6,8).

Recently, we have isolated one strain of killer yeast (*S. cerevisiae* 337) from Usina Santa Filomena, Campinas, SP., using *Torulopsis glabrata* ATCC 15126 (Killer phenotype K_{11}) as the sensitive yeast for the detection of killer yeasts. The isolated strain was examined further for its ability to exhibit killer action to the other killer yeasts (K_1 - K_{11}) by the method described in reference 3. The results indicated that *Saccharomyces cerevisiae* 337 produced killer factor resembling the K_1 phenotype. Furthermore, the strain of killer yeast showed two dsRNA (LdsRNA and MdsRNA) as shown in Fig. 1, lane 3-5. This result confirmed that *S. cerevisiae* 337 is K_1 killer yeast, which can be cured by treatment of cycloheximide and heat (Fig. 1, lane 6), disappearing band of MdsRNA.

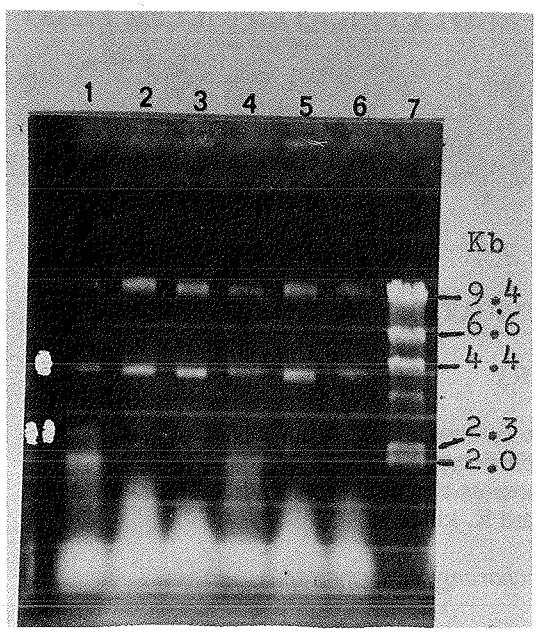
More recently, the killer activity of *S. cerevisiae* 337 was compared with standard K_1 killer yeast, using killer sensitive strain of *T. glabrata* ATCC 15126 and baker's yeast, which is used by sugarcane plants in State of São Paulo. It was found that *S. cerevisiae* 337 demonstrated higher killer activity to baker's yeast and lower killer activity to *T. glabrata* ATCC 15126 as compared to standard K_1 killer yeast (4). For this reason, we are continuing to investigate the character of the purified killer toxins from the two strains and compare the structure of MdsRNA by RNA sequencing.

RESUMO

Características de uma nova linhagem de levedura "killer"

Foi isolada uma nova linhagem de *Saccharo-*

Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos (UNICAMP)
Caixa postal 6121 - FEA, UNICAMP - Campinas, SP., Brasil - CEP 13081



Lane 1 and 4 are standard K_1 killer yeasts which showed two dsRNA.
Lane 5 is *S. cerevisae* 337, which also showed two dsRNA.
Lane 6 is *S. cerevisae* 337, which disappeared MdsRNA by heat treatment.
Lane 7 is bands of hind III DNA fragment.

FIGURE 1 - Agarose Gel Electrophoresis of *S. cerevisae* 337.

myces cerevisiae 337, de usina de cana de açúcar, que apresentou atividade "killer" semelhante ao fenótipo K_1. A eletroforese em gel de agarose mostrou que a linhagem *S. cerevisiae* 337 possui dois ds-RNAs. A atividade "killer" da linhagem foi comparada com a levedura padrão "killer" K_1.

Palavras chave: levedura "killer", plasmídeo, *Saccharomyces cerevisiae.*

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