ISSN 0001-3714

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Volume 28 Número 1 Jan.-Mar. 1997

FICHA CATALOGRÁFICA

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

Revista de Microbiologia/Sociedade Brasileira de Microbiologia.
Journal of the Brazilian Society for Microbiology
— Vol. 28, nº 1 (jan/mar 1997)
— São Paulo: SBM, [1970] -
v.:il; 27 cm

Trimestral
1970 - 1997, **1-28**
ISSN 0001-3714

1. Microbiologia I. Sociedade Brasileira de Microbiologia

NLM-QW4

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Publication of the Brazilian Society for Microbiology - São Paulo - Brazil

Papers published in Revista de Microbiologia (Journal of the Brazilian Society for Microbiology) are indexed in:

Current Contents (USA); CNRS - Centre de la Recherche Scientifique (France); Chemical Abstracts Service (USA); Cambridge Scientific Abstract (USA); Commonwealth Mycological Institute (England); Hamdard National Foundation (Pakistan); IMLA - Index Medicus Latino Americano (Brasil); Institut Nauchtoi Informatsii (ex-URSS); Periodica (Mexico); Sumários Correntes Brasileiros (Brasil); UMI - University Microfilms International (USA).

Financial support: FINEP, FAPESP and CNPq.

Printed by WINNER GRAPH (phone: (011) 5584.5753)

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REVISTA DE MICROBIOLOGIA

Volume 28 number 1 January - March 1997

Revista de Microbiologia (1997) 28:1-4
ISSN 0001-3714

RHIZOBIUM MELILOTI SURVIVAL ON PELLETED SEEDS PREINOCULATED WITH DIFFERENTLY AGED INOCULANTS

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ABSTRACT

Survival of *Rhizobium meliloti* B-36 on lucerne seeds was affected by the age of peat legume inoculants. Rhizobia death rate was similar with the different seed coats used during experiment, namely: peat, lime, perlite and perlite-soil. During a 17-27 week timespan, 90% reduction of bacteria occurred among the preinoculated seeds. However, the remaining microorganism concentration ensured a 10^4 viable cells/seed concentration.

Key words: pellet, preinoculated seeds, *Rhizobium meliloti*

INTRODUCTION

It is a well-known fact that legume inoculation is done with either powdered or liquid preparations. So far, agrotechnology is aiming to achieve a succesful seed inoculation to meet the requirements of farmers. With regard to specific, low pH types of soils or specific seeds, however -and taking into account a trend aiming to achieve preinoculated seeds (8)- many papers in literature describe different seed inoculation techniques as well as factors affecting the survival of *Rhizobium*, such as: storage temperature, type of adhesive, legume inoculant, carrier material, and strains (2; 3; 4; 7; 8; 11; 12; 13 and 14). Not much has been done, however, regarding the behaviour of rhizobia obtained from legume inoculants with a high bacteria rate but of different ages, when rhizobia interact with plants.

The *Rhizobium meliloti* B-36 survival on preinoculated lucerne seeds is described hereunder. Especifically, the effects of legume inoculant age, coupled to legume inoculant high bacterial concentration, and the seed coating material are discussed.

MATERIALS AND METHODS

Microorganisms. Commercial strains of *Rhizobium meliloti* have been kindly provided by Mr. Juan Pacheco Basurco of INTA (acronym stands for National Institute of Agrotechnology, in Spanish), from Castelar, Argentina. *Rhizobium* strains have been preserved by subculturing strains in tubes containing a yeast extract/mannitol/agar medium (15). Thereafter, tubes were kept at 5°C.

Medium. Medium used contained (g l^{-1}): sucrose, 10; yeast extract, 4; KNO_3, 0.8; $MgSO_4$. $7H_2O$, 0.2; NaCl, 0.1; $MnSO_4$. $4H_2O$, 0.006; $FeCl_3$. $6H_2O$, 0.006; K_2HPO_4, 0.5. Medium was neutralized to pH 6.8-7.0 before sterilization (1).

Liquid cultures. High cell concentrations ($1.0\text{-}2.5.10^{10}$ cell ml^{-1}) were obtained in erlenmeyer flasks (using a 1/5 liquid volume ratio per flask), in a rotary shaker at 250 rev.min^{-1}, in 36 hours. The growth temperature was set at 28°C. Inoculum represented 5-10% of the volume used in fermentation process. Initial concentration of *Rhizobium meliloti* B-36 was about 10^8 viable cell ml^{-1} in all cases.

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Legume inoculant preparation. Peat from Tierra del Fuego island, Argentina, was used as carrier material. Peat (2000 g), ground to a 200 mesh, neutralized with lime to pH 7, was autoclave presterilized at 121°C for 2 hours. Thereafter, carriers were distributed in 30 µm thickness hermetically sealed at a rate of 30 g per bag. Carriers were then sterilized at 121°C for 30 minutes. Once sterilization process was over, bags containing the carrier material were found to hold a water content of about 10%. Carriers had been inoculated up to 50-55% by adding the liquid cultures of *Rhizobium* in carriers. These preparations are called the legume inoculant.

Seeds. Lucerne seeds (Pampeano ecotype) have been kindly provided by Mr. Nestor Romero, of INTA-Anguil, Provincia de La Pampa, Argentina.

Seeds sterilization. Lucerne seeds were sterilized by firstly soaking them in 95% ethanol for 3 minutes. Thereafter seeds were soaked in acidified 0.1% $HgCl_2$ for 3 minutes. Seeds were rinsed in sterile water five or six times, and dried in a laminar flow device over a sterile filter paper. Later on, seeds were handled under sterilized conditions.

Coating materials. Peat from Tierra del Fuego island, perlite (aluminosilicate), lime and perlite-soil were used as a coating material.

Seed coating. Seeds were treated with a mixture of 5 g of legume inoculant, and 7 ml of 40% arabic gum The legume inoculant was evenly distributed over the seeds surface and either lime (30 g), or peat (6 g), perlite (8 g), or perlite-soil (8 g) was added in a shaker. Seed preinoculation was performed each week. The same legume inoculant was always used so that the survival of *Rhizobium* coming from differently aged groups could be assessed. Bags containing the coated seeds were kept at a temperature of 20-25°C.

Survival. One gram of legume inoculant was asceptically taken from every bag. Samples were put into erlenmeyer flasks along with 99 ml of distilled, sterilized water. Each erlenmeyer flask was subsequently shaken in a rotary shaker for 15 minutes. Sample was then diluted by taking 0.1 ml with a micropipette. Material was put into a tube containing 9.9 ml of distilled, sterilized water. Such a procedure was repeated as necessary so that a concentration of 30 to 300 colonies ml^{-1} could be attained. Subsamples were cultured on Petri plates and kept at 29°C (10). As regards preinoculated seeds, 20 seeds were taken from bags, and the previous, discussed steps were repeated so that the *Rhizobium meliloti* survival rate with different coating materials could be tested.

Statistics. The survival were analyzed by means of a linear regression model by simply transforming the experimental values in the base 10 logarithms. (5; 6; 9; 16 and 17).

RESULTS

The survival rate of *Rhizobium meliloti* B-36 strains on preinoculated seeds pelleted with different

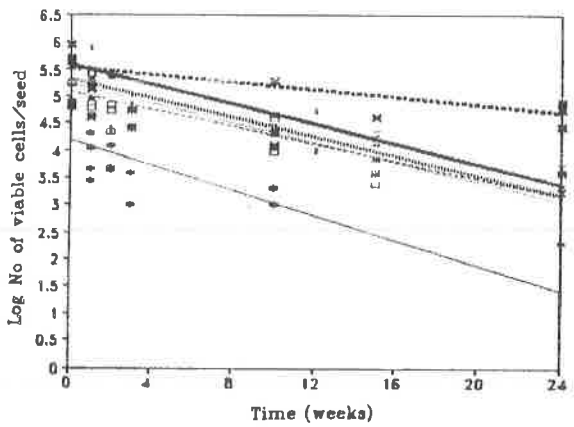

Figure 1: Influence of different physiological age on *Rhizobium meliloti* B-36 inoculant death rates on lucerne seeds coated with lime: —■— age 0 weeks (y=4.183-0.116x; r^2=0.86). ....✕..... age 1 week (y=5.208-0.089x; r^2 = 0.80). .....□..... age 2 weeks (y=5.059-0.079x; r^2=0.78). .....✕..... age 3 weeks (y=5.539-0.035x; r^2=0.79). —■— age 4 weeks (y=5.570-0.091x; r^2= 0.95). .....|..... age 15 weeks (y=5.311-0.088x; r^2=0.87).

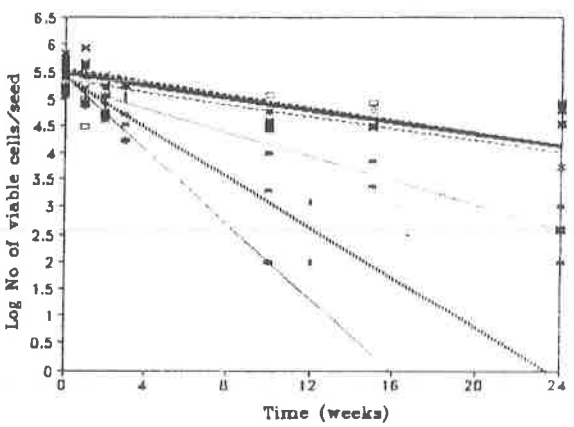

Figure 2: Influence of different physiological age on *Rhizobium meliloti* B-36 inoculant death rates on lucerne seeds coated with peat: —■— age 0 weeks (y=5.421-0.342x; r^2=0.96).....✕.....age 1 week (y = 5.260-0.112 x; r^2 = 0.85). .....□..... age 2 weeks (y= 5.340-0.052x; r^2= 0.83). .....✕..... age 3 weeks (y=5.546-0.060x; r^2=0.83). —■— age 4 weeks (y=5.456-0.056x; r^2 = 0.84). .....|..... age 15 weeks (y=5.375-0.229x; r^2=0.90).

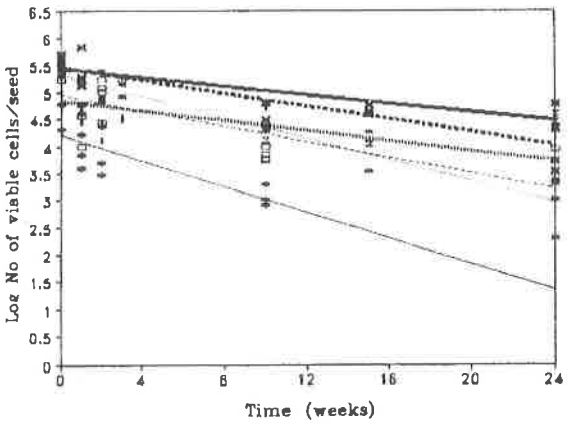

Figure 3: Influence of different physiological age on *Rhizobium meliloti* B-36 inoculant death rates on lucerne seeds coated with perlite: —■— age 0 week (y=4.792-0.197x; r^2=0.88). .....✕..... age 1 week (y=4.689-0.066x; r^2=0.85). .....□..... age 2 weeks (y=4.925-0.092x; r^2=0.84). .....✕..... age 3 weeks (y=5.379-0.052x; r^2=0.78). —■— age 4 weeks (y=5.669-0.047x; r^2=0.84). .....+..... age 15 weeks (y=5.565-0.124x; r^2=0.72).

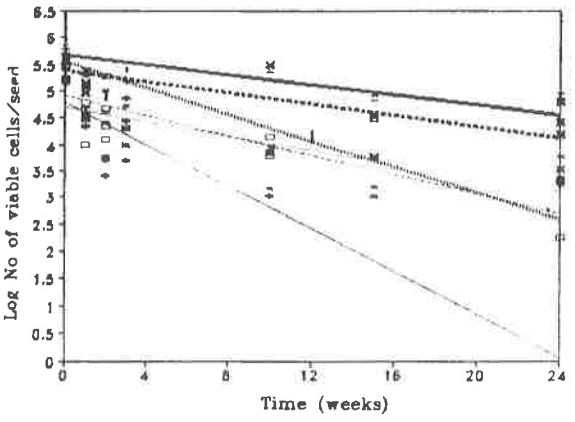

Figure 4: Influence different physiological age on *Rhizobium meliloti* B-36 inoculant death rates on lucerne seeds coated with perlite-soil: ——— age 0 weeks (y=4.214-0.119x; r^2=82.8). .....✕..... age 1 week (y=5.319- 0.096x; r^2= 0.89). .....□..... age 2 weeks (y=4.957-0.071x; r^2=0.79). .....✕..... age 3 weeks (y=5.461-0.058x; r^2=0.77). —■— age 4 weeks (y=5.425-0.393x; r^2=0.90). .....+..... age 15 weeks (y=4.857-0.045x; r^2=0.90).

coating materials can be seen in Figs. 1, 2, 3 and 4. The survival of *Rhizobium* is represented by a linear regression model (log y= a + bx) for each coating material assayed. The slope values of the curves represent death rates: it can be seen that they are inversely related to the age of the legume inoculant involved. As regards the lime coats, the lower values (i.e. a higher survival rate) were obtained with 3 weeks old legume inoculant (b= -0.035, SE= 0.010). All other coating materials used evidenced a low slope value when a 4 weeks old legume inoculant was used with either peat (b= -0.056, SE= 0.015), perlite-soil (b= -0.039, SE= 0.015) or perlite (b= -0.047, SE= 0.008). Slope estimated values were similar for all the coating materials used in the present experiment, whichever the age of legume inoculant. With regars to peat, however, death is somewhat higher.

DISCUSSION

Results suggest that whenever inoculant concentration is high (v.g. at a rate of 10^{10} viable cell g^{-1}) inoculant can be safely used over a long timespan. Age of inoculant affects favorably ther hizobia survival on pelleted seeds, at least until the third or fourth week after inoculation, even though seed response differs according to each coating type, a fact likely to be related to an adaptation of rhizobia to their new environment (osmotic pressure, humidity, etc) (14). From the results of the present experiment, it could be concluded that all the above mentioned material could be used for preparing preinoculated seeds inasmuch as a high survival rate was assessed in 17 to 27 weeks old seeds that had been treated with inoculants aged 3 to 4 weeks. Between the 17th and 27th week, preinoculated seeds evidenced 90% of population reduction -however, the concentration of the remaining microorganisms (10%) was nevertheless able to ensured a 10^4 viable cells $seed^{-1}$ concentration. Moreover, preinoculating treatment did not alter seeds germination capacity in any way.

The experiments discussed hereinabove suggest that there exists an industrial possibility to use different coats such as lime, perlite and perlite-soil, an important fact inasmuch as lime, perlite and perlite-soil are not so expensive as peat. These results give a good prospects to farmers due to the fact that our experiments have set up a higher survival level in preinoculated seeds -a rate clearly above results found in literature up to now.

RESUMO

Sobrevivência de *Rhizobium meliloti* B-36 em sementes de leucena

A sobrevivência de *Rhizobium meliloti* B-36 em sementes de leucena foi afetada pela idade dos inoculantes. A taxa de morte dos rizobios foi similar para as diferentes coberturas das sementes utilizadas

durante o experimento, discriminando: turfa, calcáreo, perlite e perlite-solo. Durante o período de 17-27 semanas, houve uma redução de 90% das bactérias presentes nas sementes pré-inoculadas. Entretanto, a concentracao restante de microrganismos garantiu uma concentração de 10^4 células viáveis por semente.

Palavras-chave: sobrevivência, *Rhizobium meliloti*, leucena.

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Revista de Microbiologia (1997) 28:5-10
ISSN 0001-3714

TEMPERATURE INCREASE AND ITS EFFECT ON MICROBIAL BIOMASS AND ACTIVITY OF TROPICAL AND TEMPERATE SOILS

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ABSTRACT

Microbial biomass and activity of tropical and temperate soils from Brazil and England, respectively, were measured during 150 days incubation at 15°C and 35°C. Extractable carbon was determined by the fumigation-extraction method giving initial biomass values ranges from 160-600 μg $C.g^{-1}$ soil for tropical soils and from 400 -1300 μg $C.g^{-1}$ soil for temperate soils. It was evident from all soils (except a tropical one) that biomass at 35°C decreased more, over a long incubation period, than at 15°C; the temperate soils had the highest decrease at 35°C. Death rate of biomass quotient (*q*D) calculated over the 150 days period of incubation showed that tropical and temperate soils, at 15°C, have similar values, but at 35°C temperate soils had higher rates than tropical soils, confirming the existence of some sensitivity of temperate soils to elevated temperature. Carbon from carbon dioxide (CO_2-C) was also measured for all soils investigated. There was a greater respiration rate at 35°C than at 15°C for all the soils. The temperate soils showed faster mineralization rate than the tropical, mainly at 35°C. At this temperature the amounts of CO_2-C respired were from 2 to 9 times as much carbon as was present in the original biomass, indicating that this carbon could only have come from the soil organic matter. Biomass specific respiration rate (mg CO_2-C evolved.g^{-1} biomass.day^{-1}) showed greater values for temperate soils at both temperatures; at 15°C the rate was 2.7 times greater and at 35°C the rate was 2.4 times greater. These results suggest that there is more available energy in the temperate soils than in the tropical soils, reinforcing that there is more carbon stored in microbial biomass of temperate soils. Comments are made on a possible contribution of microorganisms, mainly of temperate soils, to global warming, in case of a global atmospheric temperature elevation. However, it is important to consider other factors inherent to soil characteristics (i.e. organic matter added to soil, its plant cover, soil erosion, deforestation and climatic changes) as possibly stronger causes of global warming.

Key words: soil microbial biomass, soil microbial activity, global warming, tropical soil microorganisms, temperate soil microorganisms, fumigation-extraction.

INTRODUCTION

There is a worldwide concern about the expectation of a temperature increase in the biosphere and its effect on living organisms. Some investigations have showed that global warming accelerates the decomposition of soil organic matter, thus increasing CO_2 in the air, with a further increase in the global warming (see papers cited by Jenkinson *et al.*) (6).

Microbial biomass although presenting features of dormant populations, can be used as a parameter that shows changes in soil necromass, earlier than chemical

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analyses (10). Biomass carbon and CO_2 respiration rate provide useful information about living conditions of microorganisms in soil, in the absence of fresh substrate. Carbon and CO_2 measurements usually show a low and long turnover time of organic matter in soil.

It is generally accepted that (a) cryptic growth, (b) use of endocellular energy reserves, and (c) use of organic matter, are energy sources of microorganisms in the absence of fresh substrate. It is important to know which energy source prevails for the microorganisms in soil, in 'normal' temperature regimes, e.g. at 15°C and under the effect of temperature increase, 35°C.

The effect of elevated temperatures on the survival of microorganisms in the soil is an aspect studied recently by Joergensen *et al.* (7), in a grassland soil from England. These authors observed that raising the incubation temperature increased the release of substrate but also increased the specific death rate of microorganisms.

In the present investigations, realized in 1991, in the laboratory of Dr. P.C. Brookes, at Rothamsted Experimental Station (Harpenden, England), soil samples from Brazil (tropical region), supplied by the CNPSM - National Research Centre of Sorghum and Corn, of the EMBRAPA - Brazilian Enterprise for Agriculture and Cattle Raising Research (at Sete Lagoas, Minas Gerais, Brazil) and from England (temperate region) (sampled from several places in England) were incubated for 150 days, at 15°C and 35°C, and their biomass C, and CO_2 evolution were determined intermitently, to observe microbial survival in the absence of externally-supplied substrate.

MATERIAL AND METHODS

Soils selected. Three soils from Brazil (Cerrado, Jaíba and Mata) and three from England (Fosters, Silsoe and Woburn) were selected considering their similarities with respect to texture and some chemical properties. Some of their physical and chemical properties are shown in Table 1. Soils with high clay content (Cerrado and Silsoe) were chosen deliberately to observe a possible 'protection effect' of clay on microorganisms exposed to high temperatures. Soils were sampled (0 - 10cm), sieved (2mm), and discrete pieces of organic matter were removed by hand. The soil samples preparation, soils saturation capacity and their respective water content adjustment to 40% of saturation capacity, were done as described in Grisi (3). They were then stored at 5°C until experiment started.

Soils treatment. For carbon and CO_2 measurements, three replicates soil samples, 20g each (D.W. basis), were weighed into glass vials and placed into brown glass jars (c.a. 2 litres capacity). The jars with soil samples (fumigated and unfumigated) for carbon measurements contained 15ml of 1N NaOH solution, to prevent air saturation with CO_2 and 10ml of water to retain the soil humidity; the NaOH solution was renewed every 25 days. The jar with soil samples for CO_2 measurements contained 20ml of 1N NaOH solution, which was renewed 5 days after the experiment started, then on the 10th day and repeatedly every 25 days; 10ml of water was also put into each jar. All jars were stoppered with rubber bungs and were opened once a week to renew the air. The same procedure was adopted for controls. One set of three replicates for each of carbon and CO_2 measurements was incubated at 15°C and another at 35°C, and both sets were incubated for 0, 10, 25, 50, 100 and 150 days. Soil samples incubated at 35°C needed from 1 to 2ml of water after 50 days of incubation.

Analytical methods. Biomass carbon was measured by the fumigation method of Jenkinson and Powlson (5) followed by extraction, according to the fumigation-extraction method introduced by Vance *et*

Table 1 Some physical and chemical properties of Brazilian and English soils used.

Soil	pH	Saturation capacity	Clay content	Total carbon
		ml of H_2O.100 g^{-1} of soil	%	%
Cerrado (cultivated with soya and corn)	5.1	72	81.8	2.95
Jaíba (cultivated with bean and corn)	6.5	32	15.4	0.98
Mata (evergreen forest)	6.2	56	21.7	2.88
Fosters (grassland)	6.9	58	21.5	2.86
Silsoe (cultivated with cereals)	8.1	89	67.5	4.09
Woburn (grassland)	6.8	54	16.6	1.50

al. (12). The extraction method allows biomass measurements in strongly acid soils and in soil conditions where the fumigation incubation method does not work properly (3). Briefly, three replicates from each soil sample at 15°C and three from each at 35°C, of each incubation period, were fumigated in desiccator lined with moist absorbent paper and containing alcohol-free chloroform, and soda-lime in a glass vial. The desiccator was evacuated in a tap vacuum, until chloroform boiled. These samples were maintained in contact with chloroform vapour for 24h at 25°C. The unfumigated samples, in the meantime, were mixed to a 0.5M K_2SO_4 solution (1 : 4 v/v) in a shaker at 75 vibr./min for 30min (12). The fumigated samples, after the complete removal of chloroform vapour, was also mixed to the K_2SO_4 solution and their organic C extracted. Organic C in the extracts was measured in the DC 80 - Dohrmann Total Organic C Analyser, as described in Wu *et al.* (14). Biomass C rendered extractable by $CHCl_3$ was obtained by: $B_C = E_C$ X 2.2, where E_C (extracted C) = fumigated - unfumigated, as suggested by Wu *et al.*(14).

Carbon dioxide evolved and trapped in the 1N NaOH solution was measured according to the technique described by Jenkinson and Powlson (5), with some modifications, i.e., a 5ml aliquot was taken from the 20ml NaOH solution and mixed to 10ml of distilled water; CO_2 was given by the volume of a 0.5N HCl solution used to bring down pH from 8.3 to 3.7, by titration in the TTT80 Titrator (Radiometer, Copenhagen).

RESULTS AND DISCUSSION

Extractable C. All results are given per gram of oven-dry soil (/g o.-d.), except as indicated. In Fig. 1 the biomass carbon of Brazilian and English soils, over the 150 days of the experiment is shown. Initially the English soils had higher biomass (range 400-1300 μg $C.g^{-1}$ soil) than the Brazilian (range 160-600 μg $C.g^{-1}$ soil), though Woburn and Jaíba were similar. Estimations of biomass as a percentage of total soil carbon (Biomass C/Total Soil Carbon X 100) showed the following results (%): Cerrado 1.22; Jaíba 1.64; Mata 2.08; Fosters 2.57; Silsoe 3.14 and Woburn 1.18. The microbial biomass of English soils (except Woburn) seems to be a greater carbon reserve than the biomass of Brazilian soils.

We observe in Fig. 1 that at 15°C all English and Brazilian soils, except Jaíba, increased their biomasses on the 10th day, as a possible consequence of the

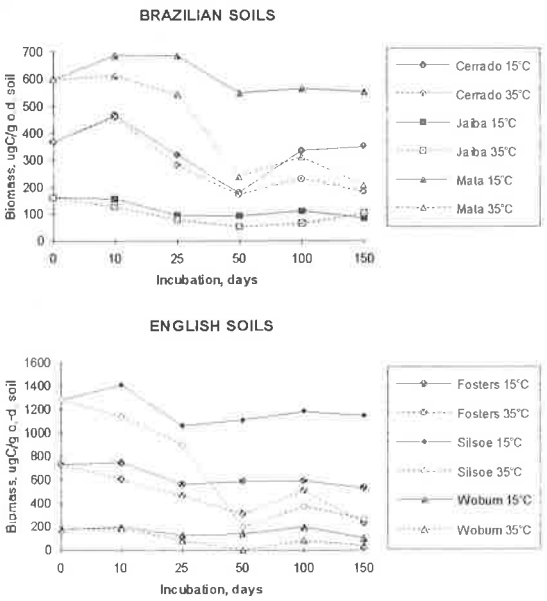

Figure 1. Biomass of carbon of Brazilian and English soils over 150 days, at 15°C and 35°C.

cryptic growth of the microbial populations. At 35°C two Brazilian soils, Mata and Cerrado also increased their biomasses on the 10th day, but most of the English soils did not increase it (Woburn did it, but not significantly). We also observe that most of the English and Brazilian soils decreased their biomasses between the days 25 and 50, partly explained because extracted C from unfumigated samples increased, mainly at 35°C; so when subtracted from the fumigated samples gave low biomass values. An overall increase in biomass C of the soils occurred between 50 and 100 days at both temperatures. This result suggests that the microbial populations are using the soil organic matter as substrate.

In the end of the incubation period most of the soils decreased their biomasses at both temperatures, except the Brazilian soils Cerrado and Mata at 15°C and Jaíba at 35°C. The final biomass C as a percentage of the initial were, at 15°C and 35°C, respectively: Cerrado 97 and 50 %; Jaíba 53 and 64; Mata 92 and 35; Fosters 72 and 32; Silsoe 89 and 21; and Woburn 59 and 21 %. Therefore it is evident from all soils, except Jaíba, that biomass at 35°C decreased more, over a long incubation period, than at 15°C; the English soils had the highest decrease at 35°C. The soil clay content does not seem to exert any protection to microbial biomass, once Jaíba (lowest clay content) had the highest biomass at the end of the incubation at 35°C and Silsoe (one of the highest clay content) had the

smallest biomass at the end of the incubation, also at 35°C. Carbon content also did not show to be a limitant factor on microbial life maintenance for a long period of incubation. Silsoe had the highest carbon content and Jaíba the smallest; this latter soil had the highest biomass at the end of incubation and Silsoe reduced it more than all the other soils.

Joergensen *et al.* (7) observed a strong decrease in biomass C at 35°C in a silty clay loam soil, from a grassland of England. Their fumigation-extraction could only be performed for the first two sampling times (25 and 50 days) in the incubation at this temperature.

The death rate of biomass, a quotient (qD) calculated over 150 days incubation, was determined as suggested by Anderson and Domsch (1), i.e., $qD = [(C_{mic})_{t1} - (C_{mic})_{t2} / (C_{mic})_{t1}] / t_2 - t_1$. The mean obtained from both sets of soils, at both temperatures (ignoring the formation of new biomass during incubation), yielded the following results: Brazilian soils, at 15°C 0.0022.day^{-1} and at 35°C 0.0075.day^{-1}; English soils, at 15°C 0.0027.day^{-1} and at 35°C 0.0217.day^{-1}. Therefore temperate and tropical soils, at 15°C, have similar death rate of biomass, but at 35°C death rate of temperate soils was higher. The mean death rate here obtained for temperate soils, at 35°C, is higher than the one obtained by Joergensen *et al.* (7) which was 0.0161.day^{-1}, but at 25°C. Anderson and Domsch (1) obtained from 28 temperate soils, at 22°C, a qD range from 0.002 to 0.01.day^{-1}. The results here obtained confirm some sensitivity of temperate soils to high temperatures.

CO_2-C measurements. Fig. 2 shows the cumulative CO_2-C evolved from Brazilian and English soils. There was a greater respiration rate at 35°C than at 15°C for all the soils investigated. The English soils showed faster mineralization rate than the Brazilian, mainly at 35°C. In Fig. 3 the respiration rates of both sets of soils are represented. The English soils again showed greater values than the Brazilian. The great respiration rate values obtained on day 50 confirmed the increase of microbial respiration of the unfumigated samples, when biomass C was measured by the fumigation-extraction method.

It is important to emphasize from cumulative CO_2-C data of Fig. 2 as a proportion of the initial biomass C presented in Fig. 1 (or ratio CO_2-C evolved: initial biomass C) that at 35°C the amounts of CO_2-C respired from both sets of soils are from 2 to 9 times as much carbon as were present in the original biomass, indicating that the amounts of carbon

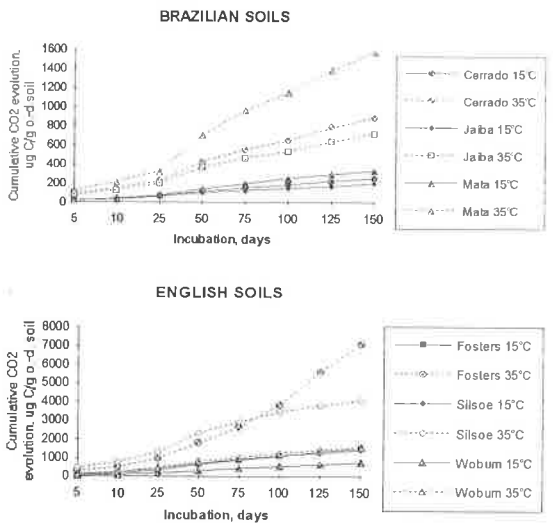

Figure 2. Cumulative evolution of CO_2-C of Brazilian and English soils over 150 days, at 15°C and 35°C.

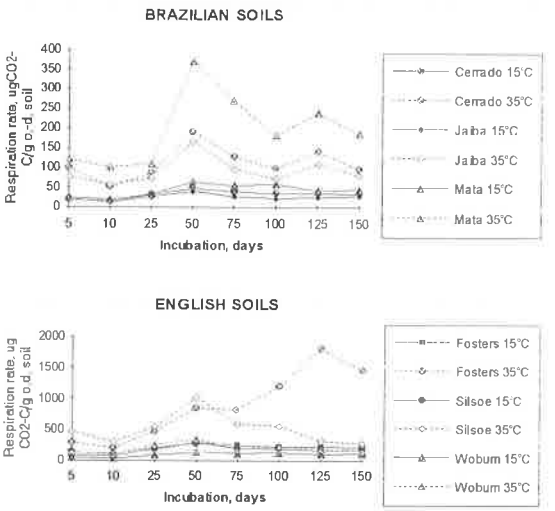

Fig. 3. Respiration rates of Brazilian and English soils over 150 days, at 15°C and 35°C.

respired could only have come from the soil organic matter.

Biomass specific respiration rate. Fig. 4 represents the biomass specific respiration rate (mg CO_2-C evolved.g^{-1} biomass.day^{-1}) of the Brazilian and English soils, respectively, at both temperatures, calculated as suggested by Joergensen *et al.* (7). It can be seen that these rates were greater for the English soils at both temperatures; and that at 15°C the rate was 2.7 times greater and at 35°C the rate was 2.4 times greater. These results suggest that there is more available energy in the temperate soils than in the

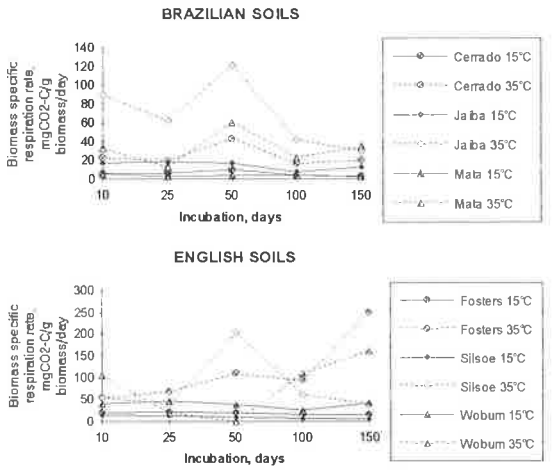

Figure 4 Biomass specific respiration rates of Brazilian and English soils over 150 days, at 15°C and 35°C.

tropical soils, substantiating the increased carbon stores observed in microbial biomass of English soils.

The results indicate that biomass maintenance and CO_2 evolved could have come from several sources: dead microbial cells or cryptic growth, endocellular reserves, and the soil organic matter; this latter seems to have been the main supply to the microbial populations, a result also obtained by Joergensen *et al.* (7).

CONCLUSIONS

The English soils showed to be a greater reserve of microbial carbon and a greater supply of available energy than the Brazilian ones. The results here obtained also suggest that microbial biomass soils from temperate regions are more susceptible to high temperature than soils from the tropics. This could be confirmed by the higher death rate biomass quotient of English soils at 35°C. This aspect supports Jenkinson's *et al.* (6) view which suggest a contribution of microorganisms to global warming, in case of a global temperature elevation. Woodwell (13) considers the stimulation of respiration, including the respiration from organic matter decay in soils as the third more important cause of atmospheric temperature elevation. Houghton *et al.* (4) also discussed this aspect. However, it is important to consider other factors inherent to soil characteristics, as organic matter added to soil and its plant cover. The organic matter dynamics in soil under climatic changes would possibly be one of the most important factors affecting the atmospheric temperature, but overgrazing, wood-gathering increase, as well as soil erosion and deforestation are the main contribution to global warming, as emphasized by Brown (2) and Salati *et al.* (11).

It is also important to consider that though temperate soils have a greater biomass, giving consequently a greater contribution to atmospheric CO_2, the microbial activity is depressed significantly for some time of the year, mainly during winter. The microbial biomass of tropical soils otherwise, will probably keep their activity steadily over the year, with small fluctuations between the rainy season (increasing it) and the dry season (decreasing it).

While temperate soils respond more intensively to air temperature increase, tropical soils do not. Some compensation therefore, with respect to the contribution of both kinds of soils to global warming, may occur.

It is important to remember that deforestation in the tropics, particularly in the Amazonian countries, and mainly in Brazil, is converting thousands of hectares of forest to pasture land for cattle ranching and small-scale agricultural settlement. The contribution of increasing rooting density, as it occurs in grassland rhizosphere, should be evaluated. Lynch and Panting (8), using intact cores of soil (in contrast to sieved soil samples used here), detected a three-fold increase in soil microbial biomass in relation to arable cropping in the same soil. As emphasized later by Lynch and Whipps (9), the carbon budgets of the grassland rhizosphere, with its strong influence on microbial biomass and activity, should be investigated. Unfortunately the methodology employed to generate reliable field data in this area, remains questionable (9), thus causing difficulties in the estimation of microbial biomass and activity and consequently their contribution to carbon cycling in the biosphere. Should this be balanced by the opposite situation in grasslands of semi-arid regions of the tropics? In this case, perennial grasses, which are less sensitive to environmental stress, are being succeeded by annual grasses, which are more sensitive and whose seeds may not germinate in dry periods (2). The contribution of rhizosphere in this situation, is presumably declining.

ACKNOWLEDGEMENTS

To Dr P.C. Brookes and Dr D.S. Powlson (head of Soil Science Dept.), for allowing me to use the facilities at Rothamsted Experimental Station. To Mrs

Carol Grace for her technical assistance. To the Federal University of Paraíba (João Pessoa, Brazil) for my leave aiming my postdoctoral with a financial support from CNPq. To Dr Robert Coler (former Professor Emeritus, University of Massachusetts) for helpful suggestions and reviewing the manuscript.

RESUMO

Elevação da temperatura e seu efeito sobre a biomassa e atividade microbianas de solos das regiões tropical e temperada

Foram determinadas a biomassa e a atividade das populações microbianas de solos tropicais e temperados, do Brasil e da Inglaterra, respectivamente, incubados por 150 dias a 15°C e 35°C. O carbono extraível foi determinado pelo método de fumigação-extração, dando valores iniciais de biomassa na faixa de 160 a 600 μg de $C.g^{-1}$ de solo (solos da região tropical) e de 400 a 1300 μg de $C.g^{-1}$ de solo (solos da região temperada). Ficou claro que a biomassa de todos os solos, exceto a de um solo tropical, diminuiu mais a 35°C do que a 15°C e que os solos temperados sofreram maior redução de biomassa a 35°C. O quociente da taxa de morte da biomassa (*q*D), calculado ao longo dos 150 dias de incubação, mostrou que a 15°C os valores de ambos os conjuntos de solo são similares, mas a 35°C os solos temperados apresentaram maiores taxas do que os tropicais, confirmando uma maior sensibilidade dos primeiros à elevação da temperatura. O carbono de dióxido de carbono ($C\text{-}CO_2$) medido em todos os solos, mostrou que os de região temperada têm taxa de mineralização mais rápida, principalmente a 35°C, onde a quantidade de $C\text{-}CO_2$ respirada foi de 2 a 9 vezes superior à quantidade existente na biomassa inicial; isto indicou que este carbono certamente proveio da matéria orgânica do solo. A taxa de respiração específica da biomassa (mg de $C\text{-}CO_2$ emanado.g^{-1} de biomassa.dia^{-1}) foi mais elevada nos solos temperados, a 15°C e 35°C. Estes resultados sugerem que há mais energia disponível para as populações microbianas nos solos temperados, reforçando o fato de que nestes solos há mais carbono armazenado na biomassa microbiana do que nos de região tropical. São feitas breves considerações sobre a possível contribuição dos microrganismos, principalmente os de solos temperados, para o aquecimento global, no caso de uma elevação da temperatura atmosférica. No entanto, outros importantes fatores inerentes às características dos solos, como matéria orgânica adicionada, cobertura vegetal, erosão do solo, desflorestamento e mudanças climáticas, são, possivelmente, causas mais fortes que precisam ser consideradas.

Palavras-chave: biomassa microbiana do solo, atividade microbiana do solo, aquecimento global, microrganismos de solos tropicais, microrganismos de solos temperados, fumigação-extração.

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Revista de Microbiologia (1997) 28:11-16
ISSN 0001-3714

BIOLOGICAL CONTROL: MICROBIAL VERSUS CHEMICAL FUNGICIDE ON GROWTH OF RICE INFECTED WITH *FUSARIUM MONILIFORME*

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ABSTRACT

Fusarium moniliforme produces giberellin, a phytotoxin with hormonal effect which causes "bakanae" disease in rice, characterized by uncontrolled growth, eventual weakness and death of the plant. In order to asses the effectiveness of disease control, a comparative study was done between metabolites produced by soil isolated bacteria (with proved in vitro activity against *F.moniliforme)* and four chemical fungicides: benomyl, triflumizole, perfurazoate and prochloraz. Rice seeds, contaminated with 14 isolates of giberellin producing *F. moniliforme*, were treated with concentrated microbial supernatant extract or chemical fungicide. Seedlings were transfered to a container with 50 g of soil, and incubated at 25°C. Continuous analysis of growth and vigour was carried out for 3 weeks and showed that all five microbial extracts efficiently inhibited the development of typical overgrowth symptom. Microbial extracts had a better response than chemical fungicides. Considering the risk of environmental contamination by chemical agrotoxic agents, the present study contributes with an additional possibility of microbial metabolite application in fungal disease control, with the advantage of avoiding undesirable chemical residues in ecosystem.

Key words: *F. moniliforme*, biological control, giberellin, chemical fungicide, "bakanae" disease

INTRODUCTION

Fusarium moniliforme produces a mycotoxin with similar effect of giberellin, a plant growth factor that has an extensive application with beneficial effect in agriculture (11, 13). The pathogenic fungus survives in the environment as the teleomorph *Giberella fujikuroi,* and causes a worldwide rice disease "bakanae", first reported in Japan in 1828 (5, 20).

Agent of variable symptoms, growth of this species in irrigated rice results in "gigantism", which manifests through thinning and lengthening of grass stems and leaves, as the consequence of giberellin action. In non irrigated land, an opposite symptom known as dwarfism occurs, due to the action of fusaric acid (3, 4, 6, 15). In the tropics, culture of mangoes are affected and giberellin causes inflorescence losses (20).

Despite of more frequent and serious problems in temperate climate crops, the optimal temperature for infection of *Giberella fujikuroi*, to develop typical symptoms of bakanae is 35°C (5).

Seed disinfection mainly through chemical treatment is the recommended method to avoid spreading the disease (15).

Benomyl is methyl 1-(butylcarbamoil)-2-benzimidazole carbamate, a wide spectrum

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fungicide recommended for rice, bean, soybean, cotton, peanut and wheat seed treatment. Its use has been extended to post-harvest decontamination of fruits such as pineapple, banana, mango and peaches (2,9,10,12,18).

Frequent application of benomyl in rice seed, selected microorganisms resistant to fungicides of the benzimidazolic group (19). In Japan they recently introduced triflumizole, perfurazoate and prochloraz, which inhibits biosynthesis of ergosterol and have already been used as agrotoxic of choice (19).

Triflumizole is an imidazolic group fungicide used in rice and wheat seeds, and extended to apple, pear, grape and peach disinfection (17).

Perfurazoate is pent-4-enyl-N-imidazol-1-ylacarbonil-DL-homoalaniate, commercialized since 1989 in Japan for rice seed decontamination and intended to be for simultaneous control of *F. moniliforme, Pyricularia oryzae* and *Cochiobulus miyabeanus* (17).

Prochloraz, another imidazolic group fungicide, is used to control "bakanae" in rice and take-all disease of wheat (16, 17).

Considering that continuous use of agrotoxics causes problems in the ecosystem, we studied the application of microbial crude extracts obtained from the culture of five selected bacteria with comproved activity against *F. moniliforme*. The effectiveness of biocontrol is compared with the activity of four chemical fungicides, recommended to control *F. moniliforme* in seedling.

MATERIALS AND METHODS

Antagonist microorganisms. Five bacteria, 3 sporulated bacilli (M11, M28 and M33) and 2 Gram-positive cocci (M31 and M34) were selected, after screening of 51 microorganisms isolated from soil, with comproved activity on *F. moniliforme* (8).

Test microorganisms. Fourteen giberellin producing *F. moniliforme*, isolated from rice bakanae disease were from the Agricultural Research Institute, Gunma.

Chemical fungicides. Benomyl, triflumizole, perfurazoate and prochloraz were used.

Crude extract. Culture supernatants of the 5 antagonist microorganisms were concentrated according to a modified Abdel-Bar *et al.* (1) method. Supernatant of 48 hours BHI culture, with 1:1 portion of ethanol was centrifuged and the alcoholic supernatant dried at 45°C. The material was resuspended with water, to a volume equivalent to 1/5 of the initial culture.

Antibiogram. Preliminar study was performed by antibiogram, as described by McKeen *et al.* (7). One hundred microliters of crude extract or chemical fungicides at 10, 100 and 1000 μg/ml were introduced in the central well (10 mm diameter), on potato dextrose agar (PDA) plate previously inoculated with *F. moniliforme* strains.

Microbial inhibitory concentrate-MIC. 50, 70, 100 and 150 μl of crude extract were homogenized with 10 ml of PDA and spread on a plate with 20 radial subdivisions. Each subdivision was inoculated with 72 hours culture of *F. moniliforme* in 0.5 cm diameter PDA. The same procedure was followed to prepare chemical fungicides plates, using 10, 100 and 1000 μg/ml of each chemical suspension and 9.0 ml of PDA.

Effect of microbial extract. Thirty grams of rice seed were contaminated with 5 ml of *F. moniliforme* culture in duplicate, by imersing the grains for 24 hours and the seeds were subdivided in 6 lots of 4 g. Each lot was treated with 5 ml of microbial extract, obtaining a total of 5 experiments and one negative control. After 24 hours of contact with microbial extract, the excess was removed by washing and the seeds incubated at 25°C. Germinated seeds were dried, placed in containers with 50 g of soil and incubated at 25°C. The growth of rice was analysed each three days, during 3 weeks by measuring leaf length in centimeters.

Effect of chemical fungicide. The same procedure was performed, using field recommended level of four chemical fungicides: benomyl at 1:1000, triflumizole at 1:300, perfurazoate at 1:200 and prochloraz at 1:100.

RESULTS AND DISCUSSION

Seed disinfection procedure, applying concentrates of microbial extracts or chemical fungicides were evaluated, by analysing development of rice previously contaminated with 14 giberellin producing *F. moniliforme*. Negative control consisted of rice cultivated without *F. moniliforme* inoculum, or microbial extract or chemical fungicide treatment. The positive control consisted of seeds inoculated with *F. moniliforme*, but without antifungal agents treatment.

Tables 1 and 2 show results of in vitro assays, while Tables 3 and 4 demonstrate respectively the effect of microbial extract and chemical fungicide on the growth of rice variety Akihikari.

Table 1. Inhibition of *F. moniliforme* by concentrates of microbial extracts.

F. moniliforme strains	Inhibition halo[a] (mm) Antagonist microorganisms				
	M11	M28	M31	M33	M34
1.1-1	20	R[b]	R	R	R
7.2-1	30	28	30	R	28
12.3-2	20	23	20	26	30
20.4-5	25	30	25	30	33
22.6-2	29	R	R	R	R
24.7-1	26	R	R	R	R
29.8-1	30	R	30	21	25
34.9-1	22	30	28	17	24
39.10-1	35	27	25	20	22
44.11-1	27	26	30	R	31
49.12-1	23	R	30	R	31
7.1	25	40	30	R	25
6.1	27	30	30	22	31
4.1	20	25	28	24	40

a: Incubation at 25°C for 40 days in PDA.
R: Resistant

Table 1 summarises the action of the 5 antagonists selected according to Motomura *et al.* (8), on giberellin producing *F. moniliforme*. The analysis after 40 days incubation showed that microorganism M11 acted as the best antagonist, controlling growth of 14 *F. moniliforme* strains. The bacteria was identified as *B. subtilis* (data not shown). *F. moniliforme* strain 1.1-1, 22.6-2 and 24.7-1 were resistant to antagonist M28, M31, M33 and M34, while strains 12.3-2, 20.4-5, 34.9-1, 39.10-1, 6.1 and 4.1 were sensitive to all five antagonist extracts. The same incubation time, for in vitro assay with chemical fungicides (Table 2) triflumizole, perfurazoate and prochloraz showed that they were less effective. Benomyl data is not in Table 2, as the tested *Fusarium* strains were resistant to this fungicide. Microbial inhibitory concentrate tests confirmed the results (data not shown), and the use of 100 µl of crude microbial extract in 10 ml of culture medium was the minimum amount required.

Biological assay confirmed that the five microbial extracts efficiently controled rice overgrowth (Table 3), which characterizes the disease caused by giberellin (3, 4, 6, 15). The average leaf length after 3 weeks in positive control was 13 cm, compared to 8.2 cm in the negative control. The average for the microbial extract treated plants were 5.6; 6.8; 6.9; 7.0 and 7.6, respectively to strains M11, M31, M34, M33 and M28. Among tested *F. moniliforme*, strain 7.2-1 was resistant to extract of antagonist M28 and M33; strain 12.3-2 to M28; strain 24.7-1 to M34 and M28.

Table 2. Inhibition of *F. moniliforme* by chemical fungicides.

F. moniliforme strains	Inhibition halo[a] (mm) Fungicide concentration (µg/ml)								
	Triflumizole			Perfurazoate			Prochloraz		
	10	100	1000	10	100	1000	10	100	1000
1.1-1	R	R	R	R	R	20	R	R	R
7.2-1	R	R	R	R	35	38	20	23	27
12.3-2	R	R	R	20	40	40	R	35	39
20.4-5	R	R	27	R	R	R	23	29	33
22.6-2	R	R	R	30	30	45	31	35	36
24.7-1	R	R	R	40	40	40	R	R	32
29.8-1	R	R	R	20	20	30	R	R	28
34.9-1	R	R	R	20	22	20	R	45	45
39.10-1	R	R	R	R	R	R	R	40	40
44.11-1	R	R	R	R	R	R	37	42	46
49.12-1	R	R	R	25	30	30	R	R	30
7.1	R	R	R	R	R	R	R	R	R
6.1	R	R	R	R	R	R	R	R	R
4.1	R	R	R	R	R	R	22	27	28

a: Incubation at 25°C for 40 days in PDA
R: Resistant

Table 3. Effect of microbial extract concentrates on the rice growth, infected with *F. moniliforme*.

F. moniliforme strains	Plant growth[a] (cm)					
	M34	M28	M11	M31	M33	Positive control
1.1-1	6.3	7.1	6.4	5.7	6.4	13.7
7.2-1	7.2	13.1	8.4	8.7	9.7	10.0
12.3-2	7.5	11.1	6.0	6.5	6.9	13.6
20.4-5	6.5	6.9	5.9	6.7	7.1	16.1
22.6-2	6.9	5.6	4.5	5.6	6.4	16.5
24.7-1	10.2	10.3	4.1	7.9	5.4	12.3
29.8-1	6.3	6.4	3.8	5.8	6.1	13.6
34.9-1	7.1	5.2	5.6	5.9	6.4	13.0
39.10-1	7.5	6.6	4.9	7.5	7.4	10.6
44.11-1	7.2	7.1	4.9	7.1	6.9	14.1
49.12-1	5.1	5.8	4.8	6.4	6.6	9.3
7.1	7.2	8.0	4.1	7.1	8.0	10.9
6.1	5.4	7.5	4.7	6.1	6.7	14.7
4.1	6.6	6.5	4.5	7.2	7.7	11.5
Average	6.9	7.6	5.6	6.8	7.0	13.0
Negative control						8.2

a: average growth, measuring 10 leaves in each assay.

Rice seeds treated with microbial extract showed no alteration in vigour and stem diameter, when compared to negative control. The best antagonist was repeated with strain M11, which inhibited all 14 *F. moniliforme* strains (Table 3). However, changes in normal plant growth, showed by 13 experiments with length of the rice lower than 8.2 cm (negative control), suggest some phytotoxicity of the metabolites produced by M11. Studies to analyse phytotoxic effect, as well as the optimal extract concentration will be run.

Table 4 shows results for the four chemical agrotoxics, using the same 14 *F. moniliforme* strains. The positive control had leaf growth of 13.8 cm in average, compared to 7.8 cm of the negative control. The results were 6.3; 7.2; 8.8 and 12.8 cm respectively, with seeds treated with prochloraz, perfurazoate, triflumizole and benomyl.

The ineffectiveness of benomyl in the bakanae disease control is reported by Wada *et al.* (19). Compared to 7.8 cm of leaf growth of the negative control, *Fusarium* contaminated plants submitted to benomyl treatment had in average 8.2 to 18.3 cm, depending on fungal strain (Table 4). These results confirm the preliminar figures obtained in vitro (Table 2). Natural frequence attaining such a level of resistence makes useless the field use of benomyl. In addition, in some countries, its carcinogenic activity has definitively prohibited its use (17,19).

Table 4. Effect of chemical fungicides on the rice growth, infected with *F. moniliforme*.

F. moniliforme strains	Plant growth[a] (cm)				
	Benomyl	Triflumizole	Perfurazoate	Prochloraz	Positive control
1.1-1	13.7	8.0	6.6	6.1	13.2
7.2-1	12.2	10.3	12.4	6.2	14.2
12.3-2	10.7	8.8	9.2	8.4	13.3
20.4-5	13.1	8.5	7.0	5.7	15.6
22.6-2	18.3	9.2	4.7	6.3	17.0
24.7-1	13.6	11.2	10.4	8.2	13.6
29.8-1	11.4	8.7	5.5	6.9	13.7
34.9-1	14.5	7.3	7.8	6.1	14.7
39.10-1	17.0	9.5	5.1	4.7	11.6
44.11-1	11.3	8.8	3.5	4.1	13.6
49.12-1	8.2	6.6	5.6	6.4	15.5
7.1	13.7	8.7	8.4	7.8	10.5
6.1	12.6	10.3	9.1	6.7	15.6
4.1	9.5	7.0	6.6	5.6	11.0
Average	12.8	8.8	7.2	6.3	13.8
Negative control					7.8

a: average growth, measuring 10 leaves in each assay.

Although triflumizole treatment resulted in rice plants with lower size than positive control (11.0 to 17.0 cm), seeds infected with 11 *F. moniliforme* strains showed leaf with 0.2 to 3.5 cm longer than negative control (7.8 cm).

Best results were obtained with prochloraz and perfurazoate (Table 4), confirming adequate requisite for use in the field (16, 17, 19). However, leaf size of only 3.5 and 4.1 cm inoculated with *Fusarium* strain 44.11-1 and treated respectively, with perfurazoate and prochloraz, indicated some degree of toxicity. If one considers that the concentrations used in this experiment are recommended levels in agriculture, the effect of toxic components should be evaluated in more detail. On the other hand, the phytotoxic action at effective concentrations of the best chemical fungicide (Table 4) suggests the advantage of microbial extracts in seed disinfection (Table 3).

In addition, the high potential of microbial extract (Table 3), requiring dilution, indicates the possibility of using diluted optimal doses, in which phytotoxic effect can be inapparent. Fungal dissemination control at field level is one of the essential points to assure good crops of a vast number of cultures of economic value (3, 4, 10, 12, 16, 17, 18).

The results of this study, obtained in plant assay, provide the feasible use of microbial metabolites in the control of fusaria disease and emphasize that the biocontrol using microbial extract demonstrated better performance than chemical antifungal agents. Furthermore, the stability of active components, even after 3 weeks, assures the plant development during germination, with the additional advantage of solving the problem of undesirable pollution by chemical residues in the ecosystem (14).

ACKNOWLEDGEMENTS

This research was supported by CAPES, CNPq and Agricultural Research Institute, Gunma, Japan.

RESUMO

Comparação entre o efeito de fungicidas biológicos e químicos no desenvolvimento de arroz inoculado com *Fusarium moniliforme*

Fusarium moniliforme, produtor de giberelina, é um fungo comumente encontrado em arroz, responsável pela doença conhecida como "bakanae", caracterizada pelo enfraquecimento e morte da planta, devido ao seu crescimento descontrolado. Para o controle da doença, testou-se extrato bruto obtido de cinco antagonistas bacterianos e quatro fungicidas, benomil, triflumizol, perfurazoato e prochloraz. Com a finalidade de avaliar a eficácia dos fungicidas, as sementes de arroz foram contaminadas com o cultivo de *F. moniliforme*, produtor de giberelinas e o arroz germinado, semeado em embalagens contendo 50 g de solo e mantidos a 25°C. A análise da viabilidade e o desenvolvimento da planta, através da medida de comprimento das folhas em intervalos de 3 dias, demonstrou que os cinco extratos microbianos inibiram o crescimento de *F. moniliforme* "in vivo". Resultado bastante promissor, já que todos os extratos obtidos de antagonistas apresentaram efeito superior ao de fungicidas químicos testados. Considerando a grande preocupação na atualidade sobre o problema cada vez mais agravante de contaminação de solo com agrotóxicos químicos, o trabalho mostrou mais uma possibilidade do emprego de microrganismos isolados de solo, capaz de minimizar o problema de resíduos químicos no ecossistema.

Palavras-chave: *F. moniliforme*, controle biológico, giberelina, fungicidas químicos, doença "bakanae".

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Revista de Microbiologia (1997) 28:17-22
ISSN 0001-3714

EFFECT OF SOY OIL ON THE CARBOHYDRATE RESERVES OF *SACCHAROMYCES UVARUM* I Z 1904 IN FERMENTATIVE CONDITIONS

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ABSTRACT

The levels of carbohydrate reserves in *Saccharomyces uvarum* I Z 1904 were quantified during culture in an HTM medium supplemented with 3 g/l of soy oil, during different fermentative cycles. The cells cultivated in this medium presented higher levels of glycogen, and a balanced behavior between the accumulation and the mobilization of trehalose in the course of fermentation cycles. The amplitude of variation in the concentration of carbohydrates during accumulation and mobilization was more homogenous in medium supplemented with lipids. This behavior regarding the reserves may be the cause of the greater viability maintained by the cells in the media with soy oil.

Key words: *Saccharomyces*, trehalose, glycogen, lipid metabolism.

INTRODUCTION

During microbial production of ethanol, minimum quantities of oxygen or an adequate quantity of unsaturated fatty acids in the fermentation media can restore the cellular activities of the yeast and the productivity in ethanol (13, 14, 15, 19, 33) since in the absence of oxygen, a condition which is brought about during fermentation, the cells are unable to grow because they do not synthesize unsaturated fatty acids and sterols.

Glycogen plays an important role in the synthesis of sterols in yeast, so there is a stoichiometric relation between its use in critical moments of fermentation, and the formation of sterols (29). Low levels of glycogen in the yeast cells limit the potential for new lipid synthesis. In the same way, deficiencies of glycogen in the inoculum were found to bring on problems related to cellular viability during fermentation, due to insufficient lipid synthesis (28).

Trehalose is a non-reducing disaccharide that occurs in great quantity in yeast and in many other fungus spores. Its intracellular concentration plays an important role in the ability of these organisms to tolerate adverse conditions of the medium, which is one of the main requirements for a good microbial performance (31). Trehalose showed to be an important membrane stabilizing factor of anhydrobiotic organisms, operating as a protection against severe dehydration and the stress caused by desiccation (5, 6, 10, 16, 17). This statement is based on the evidence that membranes can be desiccated in the presence of trehalose without losing their structural and functional integrity (22). Yeast cells in exponential growth phase on glucose contain low concentrations of trehalose (31). However, great quantities of this disaccharide accumulate in the cells in response to a heat stress (or rise in temperature) (16, 3, 34), or in the presence of high sugar concentrations

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(7, 12), which shows that trehalose is related to thermo-tolerance and osmo-tolerance.

A study on the effects of temperature and of ethanol on the composition of fatty acids and on the intracellular concentrations of trehalose in brewer's yeast showed that the exposure of cells to thermal shock (37°C), or to 10% (v/v) of ethanol for 60 min resulted in a significant increase of the intracellular concentration of trehalose (23). A similar increase in the amount of unsaturated fatty acids was observed in the cells after 24 hours of fermentation, thus suggesting that the lipids and the high concentrations of trehalose can protect the yeast against effects caused by temperature and by ethanol, which could lead to an increase in the fermentative ability.

In a previous study (9), higher average values of cellular viability were obtained in the presence of 3 g/l of soy oil, where the cells showed a viability of 77% at the end of the 6th fermentative cycle, contrasting to 65% in the non-supplemented medium. The object of this work was to study the effects of soy oil on the concentration of glycogen and trehalose of *Saccharomyces uvarum* I Z 1904, and its relation with the higher cellular viability observed in the media with soy oil.

MATERIALS AND METHODS

Microorganism - *Saccharomyces uvarum* I Z 1904 from the Departamento de Ciências e Tecnologia Agroindustrial da ESALQ - USP. The yeast was preserved in tubes with inclined solid YEPD medium, refrigerated at 5°C. Cell renewal was done before the activation of the culture, by inoculating in fresh medium and incubating at 30°C for 48 hours.

Culture activation - The culture was activated before the production phase of inoculum for the fermentations, in a medium containing (g/l): glucose, 40.0; KH_2PO_4, 5.0; NH_4Cl, 1.5; $MgSO_4$ $7H_2O$, 1.0; KCl, 1.0; yeast extract, 2.0; distilled water up to 1000 ml; pH 6.0. After growth in the maintenance medium, cell samples were transferred to 500 ml Erlenmeyer flasks, containing 100 ml of the activation medium and incubated at 30°C under shaking at 280 rpm, for 24 hours.

Dissemination of cells for inoculum - The medium used in the inoculum production phase was HTM (High Test Molasses) in a concentration of 10°Brix, supplemented with 10 g/l of yeast extract. To produce inoculum in an adequate amount for fermentation, dissemination was performed in two cycles (I and II). Aliquots of the activated culture were transferred to Erlenmeyer flasks containing the HTM medium (I), maintaining the relation of 12:100 (v/v). The number of flasks used in this phase was always equal to the number used in the fermentation one. The cultures were incubated at 30°C at 280 rpm for 24 hours, and the cellular mass obtained was asseptically separated from the medium by centrifugation (5600 x g, 10°C/ 10 min.). The cells from each one of the flasks were reinoculated into the dissemination medium (II), in a corresponding flask, and incubated as before. After 24 hours the cultures were centrifugated again, and the biomasses combined and suspended again in the total volume of medium necessary to assure homogenous inoculum for the first fermentative cycle. Then, identical volumes were distributed in fermentation flasks. The number of cells at the beginning of the fermentation was of the order of 10^8/ ml, and the cellular viability superior to 97%.

To investigate the effects of soy oil on the carbohydrate reserves, *Saccharomyces uvarum* I Z 1904 was submitted to fermentation in HTM with TRS of 18,42 g%, containing 3.0 g/l of soy oil, for 6 cycles or 6 fermentations. Control tests (without oil supplementation) were also performed.

After homogenization of the inoculum in the fermentation medium, 200 ml of this medium were distributed in 500 ml Erlenmeyer flasks in triplicate for each condition studied. Except for the control flasks, soy oil was added. The flasks were incubated at 80 rpm and 32°C for 10 hours. The cultures were then centrifuged (5600 x g, 10°C/ 10 min), and the cells suspended again in the fermentation medium, initiating the second cycle; this was repeated successively for 6 cycles.

To determine glycogen and trehalose, samples of 5 ml were taken from the cultures every two hours during the 2nd, 4th and 6th cycles, immediately frozen in liquid N_2 and stored at -25°C for further analysis. At the same time, aliquots of 5 ml were taken to evaluate the weight of the dry biomass.

Extraction of cellular trehalose - Cells from the 5 ml of culture stored at -25°C for the analysis of trehalose content were washed three times with distilled water and centrifugated at 4300 x g, 4°C/ 10 min. Trehalose was extracted from the cells according to Trevelyan and Harrison (32) and determined quantitatively by the modified (2) anthrone (21) method.

Extraction and enzymatic hydrolysis of cellular glycogen - Glycogen was extracted from the cells by

the Quain (27) method, which involves a change in the glycogen extraction procedure developed by Trevelyan and Harrison (32), followed by enzymatic hydrolysis according to Becker (4). For enzymatic determination of the glucose produced by glycogen hydrolysis, a glucose oxidase reagent was used.

RESULTS AND DISCUSSION

The average concentrations of trehalose and glycogen produced in a fermentative culture of *Saccharomyces uvarum* I Z 1904 in media with or without soy oil are shown in Table 1. Figs. 1a and 1b illustrate, respectively, the levels of glycogen and trehalose in the yeast during cultivation, under the conditions studied.

The initial data correspond to the inoculum analysis, representing the levels of glycogen and trehalose of the cells at the end of the dissemination phase. Under these conditions, the concentration of glycogen was 38 µg/mg of dry biomass, or 3.8% (Table 1). Concentrations of 2.5% were observed in *Saccharomyces cerevisiae* under similar conditions, in

Table 1: Glycogen and trehalose in *Saccharomyces uvarum* I Z 1904 cultivated in HTM without supplementation (control) and supplemented with soy oil (3 g/l), during six fermentative cycles. Average values of three experiments.

Cycle/time (h)	Glycogen µg/mg of dry biomass		Trehalose µg/mg of dry biomass	
	Control	Soy Oil	Control	Soy Oil
2nd				
2	43.20	53.80	11.70	10.30
4	40.70	56.00	10.10	13.40
6	38.20	55.60	11.90	16.00
10	30.50	53.70	12.60	16.70
4th				
2	46.20	69.30	16.60	11.10
4	47.00	68.70	19.90	14.60
6	46.70	68.00	18.40	14.00
10	45.40	73.20	23.50	13.20
6th				
2	63.40	67.30	12.70	9.00
4	66.90	64.20	11.10	12.30
6	79.20	64.40	10.80	12.70
10	85.40	58.70	9.70	11.90

Initial data: Glycogen = 38.20 g/mg dry biomass; Trehalose = 10.60 µg/mg dry biomass.
TRS in media = 18.42 g/100ml; T°of cultivation = 32°C; Agitation = 80 rpm

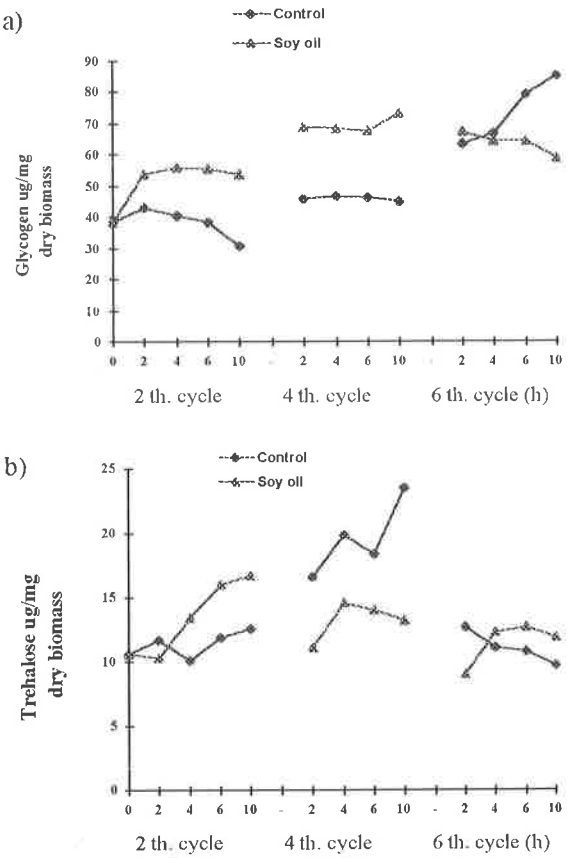

Figure 1: Levels of glycogen (a) and trehalose (b) in *Saccharomyces uvarum* I Z 1904 cultivated for six fermentative cycles in HTM non-supplemented with lipids (control) and supplemented with soy oil (3 g/l).

complete media (20). In the case of beer fermentation, Quain *et al.* (29) state that glycogen concentrations of 1.6 to 2.0% in the yeast inoculum are necessary to assure satisfactory fermentation, which are lower amounts than those observed in this study. However, during brewing the yeast is submitted to only one cultivation, and although this goes for a longer period than those in the industrial production of ethanol, the cells are not forced to work at their limit of tolerance to alcohol, as in the industrial production of ethanol, due to the successive recyclings to which they are submitted. Under those conditions, one can therefore admit that a good performance of the yeast depends on accumulation mechanisms and reserve mobilization.

A progressive increase in the concentration of glycogen in the cells cultivated in HTM supplemented with soy oil was observed until the fourth cycle when the cells were transferred from the dissemination phase to fermentative conditions (Table 1; Fig. 1a). At the

end of this period, the levels of glycogen, 73.2 μg/mg of dry biomass, corresponded to twice the amount observed in the inoculum, and no great variations were observed during the 10 hours of fermentation.

On the other hand, during the sixth cycle there was a gradual increase in the concentration of glycogen in the cells cultivated without lipid supplementation (Table 1; Fig.1) and a decline in the concentration of glycogen in the cells cultivated in the presence of soy oil, but without dropping to levels below the ones in the second cycle. In any case, in fermentative conditions, the cells maintained glycogen concentrations superior to those shown in dissemination conditions. These results are in accord with the interpretation of Lillie and Pringle (20), who claim that the accumulation of glycogen occurs in response to the limitation of nutrients, since in our tests the yeast was transferred from the dissemination phase, where the media was supplemented with yeast extract, to a fermentation medium without supplementation.

Quain *et al.* (29) verified the existence of a stoichiometric relation between glycogen oxidation and the formation of sterols in brewer's yeast, suggesting that low levels of glycogen can limit the potential for new lipid synthesis. The authors also verified that low glycogen levels in the inoculum created problems in relation to cellular viability during fermentation, due to insufficient lipid synthesis (28). Since in preceding works (1, 9) an increase of the viability of *Saccharomyces uvarum* IZ 1904 cultivated in the presence of soy oil was verified, our intention was to establish its relationship to the content of glycogen in the cells. The data show that cells cultivated in the presence of soy oil present higher levels of glycogen than the control cells till the end of the fourth fermentative cycle; this fact may be connected with the higher cellular viability observed under these conditions. The increase of the amount of glycogen in the control cells in the sixth cycle did not reflect concomitant changes in the parameters studied.

Mechanisms of accumulation and glycogen mobilization similar to those in this work were verified with *Saccharomyces cerevisiae* (NCYC 240) (29), where the accumulation of carbohydrates in the cells during the most active phases of beer fermentation, between 12 and 17 hours, was observed. During the last phases of fermentation, when the yeast ceased growth but needed energy to keep cellular functions, the glycogen reserves slowly decreased.

The accumulation of reserves was not identical for both carbohydrates. The levels of trehalose were quite lower than the glycogen levels during all the cycles (Table 1; Fig. 1b). Lower trehalose accumulation than glycogen accumulation was observed before, which shows that the two carbohydrates perform different physiological roles (20, 29). Trehalose accumulated in cells that received soy oil supplementation in an identical manner in the analyzed cycles, that is, the yeast showed a lower trehalose concentration at the beginning of each cycle. At the end of the sixth cycle, the concentration of trehalose kept close to that found in the inoculum.

Cells cultivated in media not supplemented with lipids (control) accumulated higher amounts of trehalose in the fourth cycle; concentrations around 25 μg/mg of dry biomass were detected after 10 hours of fermentation. During the sixth cycle, the degradation of trehalose in the cells coincided with the glycogen increase, indicating that there was not a concomitant synthesis of the two reserves at that moment. The content of trehalose in yeast cells can vary from 1 to 20% of the dry cellular weight due to nutritional factors or different stages of the life cycle (18, 25, 20), which shows that the mechanisms that govern the regulation of trehalose metabolism are quite complex. Many results in the literature indicate that the majority of yeast strains require efficient aeration to accumulate trehalose (30) and, according to Panek (24), anaerobic conditions reduce the amount of trehalose accumulated by yeast since the energy formed under these conditions does not permit storage of this reserve. The low trehalose levels, from 0.9 to 1.67%, observed for *Saccharomyces uvarum* I Z 1904 in the fermentative conditions, correspond to those observed before for the same strain (11, 8). In those tests, levels of trehalose from 1 to 4% for this yeast were registered. *Saccharomyces cerevisiae* M-33-A and baker's yeast *Saccharomyces cerevisiae*, also used in the industrial production of ethanol, presented proportions of 8 and 6% of trehalose respectively. The authors also observed that, although *Saccharomyces uvarum* I Z 1904 showed low concentrations of trehalose, this did not incur in differences in viability of this strain.

Panek (26) mentions that under stress conditions, as occur during the production of ethanol, where the cells are recycled successively, the improvement of cellular viability by a high content of trehalose is of great importance. Using the results obtained in this study, no correlation was established between the greater viability maintained by cultivated cells in the presence of soy oil and trehalose concentration.

However, the data indicate that yeasts showing greater viability in media supplemented with lipids may be related to the balanced behavior shown by the cells, regarding the accumulation and the mobilization of this reserve (Table 1; Fig. 1b). Panek (25) demonstrated that synthesis and mobilization of trehalose in precarious nutritional conditions occur in cycles. However, a good balance between synthesis and mobilization favors cellular viability. Our results indicate that the concentration of trehalose in the cells that received soy oil supplementation decreased during the manipulation of the biomass between one recycling and the next one when the yeast was exposed to air, and gradually increased in the course of fermentation.

Another important observation is that the range of the concentration of the carbohydrate reserves shown by the cells during accumulation and mobilization was processed in an interval which was more homogenous when the medium was supplemented with lipids.

RESUMO

Efeito do óleo de soja sobre os carboidratos de reserva de *Saccharomyces uvarum* I Z 1904 em condições fermentativas

Os níveis de carboidratos de reserva da levedura *Saccharomyces uvarum* I Z 1904 foram quantificados durante cultivo em meio de HTM suplementado com 3 g/l de óleo de soja, no decorrer de diferentes ciclos fermentativos. As células cultivadas no meio contendo óleo apresentaram níveis mais elevados de glicogênio e um comportamento equilibrado entre a acumulação e mobilização da trealose no decorrer dos ciclos. A amplitude de variação na concentração dos carboidratos de reserva exibida tanto durante a acumulação como durante a mobilização se processou dentro de uma faixa mais homogênea quando o meio foi suplementado com lipídios. Este comportamento em relação às reservas pode ser a causa da maior viabilidade mantida por células cultivadas na presença de óleo de soja.

Palavras-chave: *Saccharomyces*, trealose, glicogênio, metabolismo de lipídios

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Revista de Microbiologia (1997) 28:23-24
ISSN 0001-3714

UTILIZATION OF *SPIRILLUM VOLUTANS* FOR MONITORING THE TOXICITY OF EFFLUENTS OF A CELLULOSE AND PAPER INDUSTRY

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ABSTRACT

The motility of Spirillum *volutans* was used for monitoring the toxicity of effluents of a cellulose and paper industry. Results indicated that there was no correlation between organic content and the toxic effects of the residues in the effluents. The effluents from the chlorination step and from the sludge ponds presented the highest toxicity. On the other hand, the final effluent from the biological treatment basin had no toxic agent. This bioassay showed to be a simple and reliable technique that can be used for adequately monitoring the toxicity of effluents.

Key-words: *Spirillum volutans*, toxicity, cellulose, paper.

INTRODUCTION

Toxicity assays are a well known and important tool in evaluating biological effects of environmental pollutants. The use of microorganisms to indicate toxic substances in the environment has become a promising technique (7, 8), since it is fast and sensitive. As the results of the assays are obtained in a short time, the effluent disposal can be stopped immediately, avoiding environmental damages (3). The successful use of bacteria is due to the high degree of membrane structures that present the same biochemical characteristics of the higher organisms (1). Among microorganims, bacterium cells, particularly *S. volutans*, are good toxicity indicators.

The goal of this study was to evaluate the use of *S. volutans*, a strain of aquatic bacteria with a rotating fascicle of flagella at each pole, as an indicator of toxicity of effluents of a cellulose and paper industry.

MATERIALS AND METHODS

Microorganisms and culture medium: *Spirillum volutans*, strain ATCC 19554 was utilized in this work. The bacteria were grown in semi-solid Bacto Casitose Succinate Salts Medium (BCSS) at 25°C for 48h, as described by Goatcher *et al.* (5).

Effluent samples: The effluent samples were obtained from a cellulose and paper industry that produces 650 t/day of cellulose by Kraft process and 450 t/day of paper. Effluents were taken from the following production points: wood washing and grinding, cellulose bleaching (chlorination, oxidadtive extraction and hypochlorination), gas washing (gases from the wood digestion) and water resulting from machinery washing. All these effluents and the residual waters from two different sludge ponds (sludge pond-1 and sludge pond-2) were mixed in the primary decanter. These effluents were pumped to the biological treatment basin (basin 1) for a 4-day retention time. Finally, the supernatant of this basin was transferred to the second treatment basin (basin 2) for a 5-day retention time and then to the receiver stream (9). Effluent samples were collected every hour during 24 hours. After this, all samples were mixed and called "composite effluent sample". This procedure was done twice.

Toxicity assays: The bioassay was carried out according to Bowdre and Krieg (2), modified by Goatcher *et al* (5). In this assay, the concentration of

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the toxic agent is determined based on the disturbance of the typical motility of at least 90% of *S. volutans* cells after 30 minutes of exposure (MEC $_{90}$,30 min).

Organic content evaluation: The organic content of the effluents was determined by COD and BOD determinations, according to Standard Methods for Examination of Water and Wastewater, 1989.

RESULTS AND DISCUSSION

The bioassay results are shown in Table 1. Regarding the effluent generation points, it was confirmed that chlorination is the step responsible for the largest organic pollutant content and the highest toxicity (MEC 90, 30 min = 1%). In this step, organochlorinated compounds and lignin were formed. These substances have important biocide activity (4, 6, 10, 11). As expected, the effluents of the wood washing and grinding points and the condensed effluents of the evaporation step and gas washing had low organic content and low toxicity or none((MEC $_{90}$,30 min = 90 and 95%). However, the effluents of sludge ponds 1 and 2 were highly toxic, despite their low organic content. Another interesting result, shown in Table 1, is the efficiency of the biological treatment: the final effluent of biological basin 2 was non-toxic and had low organic content.

CONCLUSIONS

This study demonstrated that utilization of *S. volutans* as an indicator of pollutant toxicity is a reliable method to detect sources of pollution in effluents produced in several steps of the process of a cellulose and paper industry. Using this bioassay, the lack of correlation between organic content and toxic effects was also demonstrated. The importance of the toxicity parameter over the determination of the organic content of an effluent for pollution monitoring became evident. Finally, it should be mentioned that the utilization of sublethal responses increases the sensitivity of the detection of toxic agents in effluents. Since this method requires no previous treatment of the effluent, it can be used in all laboratories.

ACKNOWLEDGEMENTS

The authors wish to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support, as well as Indústria de Papel e Celulose RIPASA and Companhia de Saneamento Básico do Estado de São Paulo (CETESB) for providing the strain of *S. volutans*.

RESUMO

Utilização de *Spirillum volutans* para monitoramento da toxicidade de efluentes em uma indústria de papel e celulose

A motilidade da bactéria *Spirillum volutans* foi utilizada para o monitoramento da toxicidade dos efluentes de uma indústria de papel e celulose. Os resultados demonstraram não haver correlação entre o conteúdo orgânico e os efeitos tóxicos dos resíduos. Além disso, verificou-se que os efluentes da etapa de cloração e das lagoas de lama foram os mais tóxicos e que o efluente do tratamento biológico final não apresentou toxicidade. O bioensaio apresentou-se adequado ao monitoramento destes efluentes.

Palavras-chave: *Spirillum volutans*, toxicidade, celulose, papel.

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Revista de Microbiologia (1997) 28:25-31
ISSN 0001-3714

EFFECTS OF NUTRITIONAL FACTORS ON GROWTH OF *LACTOBACILLUS FERMENTUM* MIXED WITH *SACCHAROMYCES CEREVISIAE* IN ALCOHOLIC FERMENTATION

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ABSTRACT

The growth of *Lactobacillus fermentum* was studied in mixed culture with *Saccharomyces cerevisiae* during alcoholic fermentation of high test molasses (HTM).Yeast extract or a group of 17 amino acids caused a strong and fast decrease in yeast viability due to the strong increase of acidity produced by bacteria. Pure culture of *Lactobacillus fermentum* in dry sugar cane broth confirmed amino acids as the main nutrients needed to stimulate the growth of bacterial contaminant during alcoholic fermentation. The absence of *L. fermentum* growth was obtained when leucine, isoleucine or valine were not added to the medium. Phenylalanine, alanine, glutamic acid, cystine, proline, histidine, arginine, threonine, tryptophane, serine and methionine inhibited the bacterial growth at least in one of the cultures of *L. fermentum* tested.

Key words: amino acids, stimulation, growth, *Lactobacillus fermentum*, *Saccharomyces cerevisiae*, mixed culture.

INTRODUCTION

Lactic acid bacteria (*Lactobacillus* and *Leuconostoc)* are a common group of yeast alcoholic fermentation contaminants and are frequently associated with process problems. (8,17). In Brazil, the industrial production process of fuel alcohol uses yeast cell recycling. Microbial contaminants are also recycled and this may cause many problems due to bacterial growth that competes with the yeasts for the substrate. The *Lactobacilli* are adapted to alcoholic fermentation conditions (18), but *Leuconostoc* is more sensitive to them (10). *Lactobacillus* causes serious problems in the alcoholic fermentation. *Lactobacillus fermentum* is one of the bacteria responsible for yeast flocculation (21,24).

The antagonism between *Lactobacillus* and *Saccharomyces cerevisiae* is due to organic acids produced by the bacterial cells. Lactic acid can strongly inhibit yeast metabolism and make decrease the alcoholic yield of the yeast cells. Essia-Ngang *et al.* (5) observed a 30% decrease in ethanol yield in yeast fermentation of beet sugar, with a production of 5 g/l of lactic acid by lactic acid bacteria contamination. Maiorella *et al* (14) noted an 80% reduction in the yeast population in the presence of 40 g /l of lactic acid.

Alcoholic fermentation and yeast viability are strongly inhibited by *L. fermentum* after a few cycles in a fed-batch process with cell recycling and without any bacterial control. There is a significant decrease in alcoholic yield (18) when the total acidity expressed in lactic acid / l of wort is above 6.0g.

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Lactic acid bacteria grow only in complex media and have nutritional requirements for amino acids, peptides, vitamins, salts, fatty acids, carbohydrates and others (9). Several nutrients have been proposed for the growth stimulation of lactic acid bacteria during alcoholic fermentation. Amino acids released by yeast autolysis (22), monosaccharides liberated during sucrose hydrolysis by yeast (6) and ethanol in a concentration lower than 2% (11) have been detected to stimulate bacterial growth during mixed culture with yeast in alcoholic fermentation process. The present work shows the results of the study of the main nutrients necessary for the stimulation of *L. fermentum* in ethanolic fermentation by *S. cerevisiae* from commercial baker's yeast in a fed-batch process with cell recycling.

MATERIALS AND METHODS

Microorganisms. *Saccharomyces cerevisiae* obtained from baker's yeast (Prod. Alim. Fleishmann Royal Ltda.) was isolated in a PDA (Potato Dextrose Agar) medium (Difco). The yeast biomass was obtained from a culture in a medium containing 2.0% (v/v) sugar cane molasses, 0.62% (w/v) ammonium sulphate (Cinética Química Ltda.) and 0.17% (w/v) ammonium phosphate (Cinética Química Ltda.).The yeast was incubated at 30°C for 24 h in a temperature-controlled shaker (Tecnal mod. TE 420) operated at 120 rpm. The yeast cells were washed and used in alcoholic fermentation tests.

Three of the cultures of *Lactobacillus fermentum* used in this study were obtained from the Culture Collection of Fundação Tropical de Pesquisas e Tecnologia André Tosello - Campinas - Brazil. *L. fermentum* CCT 1400 and 1407 were isolated in Brazilian distilleries with serious bacterial contamination problems, and *L. fermentum* CCT 0559 came from ATCC (American Type Culture Collection). For the alcoholic fermentation, only *L. fermentum* CCT 1407, a non-flocculating culture, very resistant to alcoholic fermentation conditions, was used (18).

Fermentation conditions. The alcoholic fermentations were carried out in a shaker (Tecnal mod. TE 420) as a stirred (120 rpm) fed-batch process with cell recycling at 32°C. The medium was prepared with high test molasses diluted in water (18°Brix), supplemented with 0.5 g/l urea and pH adjusted to 5.5 with 0.1 N HCl. The substrate was fed in two steps: 250 ml were added at the beginning of the process and additional 250 ml were added after 2 hours. The fermentation process was interrupted after 20 hours.

Yeast extract (Difco) and 17 amino acids were added to the medium at the beginning of the fermentation. The amino acids were analytical grade from E. Merck (Darmstadt, Germany) and consisted of: DL-alanine (Ala - 0.400 g/l), L-arginine (Arg - 0.484 g/l), L-aspartic acid (Asp - 0.200 g/l), L-histidine (His - 0.124 g/l), DL-phenylalanine (Phe - 200 g/l), L-proline (Pro - 0.200 g/l), DL-serine (Ser - 0.100 g/l), DL-threonine (Thr -0.400 g/l), DL-tryptophane (Trp - 0.0800 g/l), L-tyrosine (Tyr - 0.200 g/l), DL-valine (Val - 0.500 g/l), L-leucine (Leu - 0.250 g/l), DL-isoleucine (Ile - 0.500 g/l), DL-methionine (Met - 0.400 g/l), L-histidine (His - 0.200 g/l), L-cysteine (Cys - 0.100 g/l) and L-glutamic acid (Glu - 0.600 g/l).

The inoculum for the first cycle consisted of 35 g (wet wt) *S. cerevisiae* from baker's yeast prepared as described before and mixed with 0.70 g (wet wt) of *L. fermentum* CCT 1407 grown in MRS medium (Difco). Both were suspended in 200 ml distilled water. For the following cycles, yeast and bacterial cells were centrifuged (International Equip. Mod. B-20A) at 4°C at 2200 x g for 30 min, and the cells were resuspended in distilled water.

Yeast viability and budding were determined with fresh samples (2ml) from a 9-hour fermentation culture using methylene blue staining technique (12). Counts of viable bacteria and yeasts were determined with 1 ml fresh inoculum samples by subculture on MRS agar (Difco) with 0.01% (w/v) cycloheximide (Sigma Chem. Co., St. Louis, MO) and on Standard Plate Count agar (Difco) containing 0.01% (w/v) chloramphenicol (Carlo Erba, Brazil) and 0.01% (w/v) tetracycline (Sanval, Brazil), respectively. At the end of fermentation, the total acidity in the samples of cell-free fermented broth was determined by titration with 0.1N NaOH. The pH of the samples was also determined using standard methods (2).

The alcoholic fermentation was assayed through four repetitions and the results presented are an average of the four analyses.

Nutritional requirements of *Lactobacillus fermentum*. Nutritional requirements of *L. fermentum* were determined using 4% (w/w) dry sugar cane broth (dscb) in distilled water, supplemented with the following nutrients (1):

- vitamins (Sigma Co., Mo, USA): pyridoxine hydrochloride (2.0 μg/ml) , nicotinic acid (2.0 μg/ml), thiamine (1.0 μg/ml) , calcium pantothenate (1.0

µg/ml), p-amino-benzoic acid (0.2 µg/ml) and folic acid (0.02 µg/ml).

- salts (Cinética Química Ltda, Brazil) : $MnSO_4.1H_2O$ (0.63 g/l) , $MgSO_4.7H_2O$ (2.33 g/l), sodium acetate (57.1 g/l) and ammonium citrate (22.9 µg/l).

- amino acids: as described above.

The test was performed by five successive transfers of a 0.05 ml fresh inoculum of *L. fermentum* CCT 1407, CCT 1400 and CCT 0559, in 6 ml of medium incubated for 72 h at 30°C. The cell growth was determined in aseptic conditions by measuring the turbidity at 600 nm after a 72-hour incubation.

RESULTS

The addition of 10 g/l yeast extract to the medium of *S. cerevisiae* alcoholic fermentation mixed with *L. fermentum* caused a fast decrease in the percentage of yeast viability and live buds and a strong increase in the broth acidity (approximately twice the control) in the last cycle (Table 1). Yeast viability, buds and yeast number decreased considerably after the 3rd cycle with yeast extract treatment, while the control showed nearly 60% viable cells and only a little change in other parameters even after 6 cycles. The acidity of the broth and the number of bacteria increased with the yeast extract treatment, respectively 2.0 and 3.0 times, when compared to the control in the 3rd cycle (Table 1 and Fig. 1).

The addition of 17 pure amino acids to the medium in the first cycle caused a similar bacterial growth pattern when compared to the yeast extract treament, but to a lesser extent (Table 2). Yeast viability decreased to 54% in the 6th cycle while in the control it was 85%. Also the acidity of fermented broth in the 6th cycle increased quickly (2.9 times the control), and from the 7th to the 9th cycle the acidity increased and was close to the results obtained through the yeast extract treatment (about 15 g/l).

The amount of live yeast buds in the 17-amino acid treatment remained around 6% in the 9th cycle (Table 2) and they became smaller in size and longer in shape (data not shown). Similar morphological observations were noted with yeast extract treatment when the live buds increased from zero to 1.3% in 5th to 6th cycle, respectively.

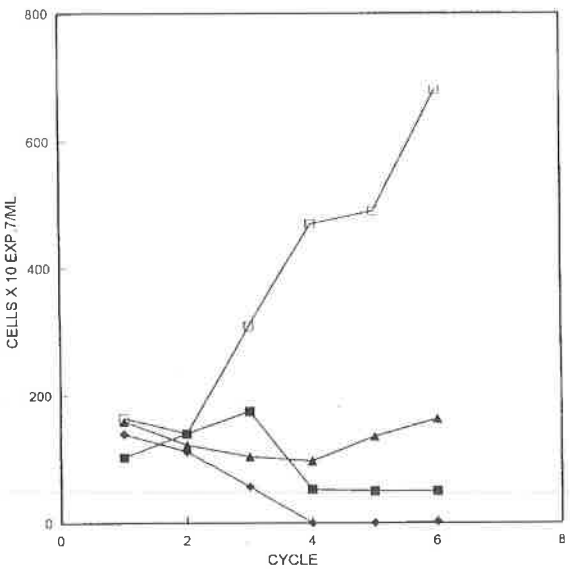

Figure 1. Effect of 10 g/l yeast extract added to the alcoholic fermentation medium on the yeast and bacterial numbers, expressed in viable colonies x 10^7/ml inoculum.
■ yeast number-control,
▲ bacterial number-control,
♦ yeast number-yeast extract,
⊟ bacterial number-yeast extrat.

Table 1. Averages of viability and buds of yeast cells, acidity and pH in yeast alcoholic fermentation of diluted high titer molasses supplemented with yeast extract.

cycle	yeast viability (%)		alive buds (%)		acidity (g/l)		pH	
	contr*	treat**	contr.	treat.	contr.	treat.	contr.	treat.
1	96	90	8.5	54	5.07	5.13	3.21	3.58
2	85	41	8.0	6.8	5.39	7.19	3.28	3.51
3	69	16	19	5.4	6.16	12.1	3.25	2.91
4	61	<5	19	0	5.95	14.3	3.01	2.51
5	56	<5	14	0	6.89	14.5	2.88	2.10
6	55	<5	13	1.3	-	-	2.81	2.09

* = control;
** = treatment of yeast extract.

Table 2. Averages of viability and buds of yeast cells, acidity and pH in yeast alcoholic fermentation of diluted high titer molasses supplemented with 17 pure amino acids.

cycle (20 h.)	yeast viability (%)		alive buds (%)		acidity (g/l)		pH	
	contr*	treat**	contr.	treat.	contr.	treat.	contr.	treat.
1	98	96	30	25	3.93	3.99	4.08	4.10
2	98	88	16	14	3.47	4.08	4.28	4.38
3	96	83	6.4	9.1	3.12	4.32	4.28	4.38
4	90	75	11	11	3.40	5316	4.38	4.34
6	85	54	8.8	5.6	4.38	12.6	4.13	3.50
7	74	40	7.6	9.9	5.74	15.1	3.93	3.36
8	67	31	8.6	6.2	5.97	15.1	3.84	3.36
9	68	27	7.3	6.1	6.83	14.4	-	-

* = control;
** = treatment of yeast extract.

The bacterial population increased sharply in the 5th cycle, as shown in Fig. 2. This increase indicated that the growth of the bacteria occurred during the 4th cycle, since the sample was taken from the inoculum. The population increase continued up to the 7th cycle, reaching 5.6 x 10^9 viable colonies/ml, while the control presented 2.4 x 10^8 viable colonies/ml only.

Pure culture of *L. fermentum* in dscb with several nutrients (Table 3) confirmed the stimulation effect on bacterial growth by yeast extract and by the group of 17 amino acids and the lack of effect by vitamins and salts. The best stimulation was obtained through the combination of all these nutrients. Partial combination of 7 to 9 amino acids added to dscb medium did not fully support the growth of *L. fermentum*. A combination of the following amino acids Cys, Glu, Met, Trp, Val, Leu and Ile, or Ala, His, Phe, Pro, Asp, Ser, Arg, Tyr, Lys, added to dscb, supported only a slight growth of the bacteria.

All but one of the 17 amino acids listed in Table 4 were tested in dscb. Amino acids were grouped in 3 classes: the first one, which comprised Tyr, Asp or Lys, did not affect the growth of any of the 3 tested cultures; the second one caused a complete absence of *L. fermentum* growth after 48 hours, as shown by Leu, Ile and Val; and the third one showed some degree of growth inhibition when compared to the control with 17 amino acids in 18 or 48 hours. Phe, Ala, Glu, Cys, Pro, His, Arg, Thr, Trp, Ser, Met belonged to this last class. The three tested cultures of *L. fermentum* presented a similar behavior in regard to the first or second class of amino acids, but the inhibition by the amino acids of the third class varied among the cultures.

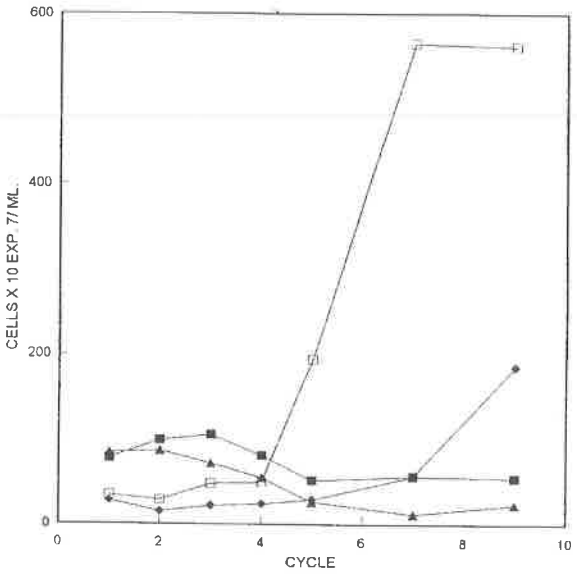

Figure 2. Effect of 17 pure amino acids added to the alcoholic fermentation medium on the yeast and bacterial numbers, expressed in viable colonies x 10^7/ml inocolum.
■ yeast number-control,
▲ bacterial number-control,
♦ yeast number- 17 amino acids treatment,
⊟ bacterial number-17 amino acids treatment.

DISCUSSION

The results of the mixed culture of *S. cerevisiae* and *L. fermentum* performed accordingly to other works (18,19) and confirmed the competition of these microorganisms. In the first 4 cycles, bacteria suffered a growth inhibition (Figs. 1 and 2) in the control, in spite of the introduction of an adapted high viable inoculum in the first cycle. After some cycles, yeast growth was inhibited by the acidity produced by

Table 3. Evaluation of *Lactobacillus fermentum* growth in a medium containing dry sugar cane broth (dscb) supplemented with several nutrients and incubated at 30°C for 72 h.

nutrients	*Lactobacillus* fermentum CCT		
	1400	1407	0559
dscb	-	-	-
dscb and salts	-	-	-
dscb and vitamins	-	-	-
dscb and yeast extract	++++	++++	++++
dscb and aa[1] and salts	+++	+++	+++
dscb and aa[2] and salts	+	-	-
dscb and aa[3] and salts	-	-	-
dscb and aa[1] and vit	++++	+++	+++
dscb and aa[1] and vit and salts	++++	++++	++++

- = absence of absorbancy
++ = absorbancy 0.10 - 0.18
+++ = absorbancy 0.19 - 0.24
++++ = absorbancy > 0.25.
aa[1] = pool of 17 amino acids
aa[2] = Cys, Glu, Met, Trp, Val, Leu, Ile.
aa[3] = Ala, His,Phe, Pro, Asp, Ser, Arg, Tyr, Lys. vit - vitamins

Table 4. *Lactobacillus fermentum* growth in a medium containing dry sugar cane broth (dscb) supplemented with 16 amino acids excluded the one listed (incubation at 30°C for 72 h)

class* amino acid excluded	*Lactobacillus fermentum*					
	CCT 1400		CCT 1407		CCT 0559	
	18h.	48h.	18h.	48h.	18h.	48h.
aa[1]	+++	+++	+	+++	+++	+++
1st Tyr	+++	+++	+	+++	+++	+++
Asp	+++	+++	+++	+++	+++	+++
Lys	+++	+++	+++	+++	+++	+++
2nd Ile	-	-	-	-	-	-
Val	-	-	-	-	-	-
Leu	-	-	-	-	-	-
3rd Phe	+	+++	-	+++	-	+++
Ala	-	+	-	+++	-	+
Glu	-	+++	-	+	-	+++
Pro	+++	+++	-	+++	+++	+++
His	+++	+++	-	-	+++	+++
Arg	+++	+++	-	+++	+++	+++
Thr	-	+++	-	+	-	+++
Trp	+++	+++	-	+++	+++	+++
Ser	+++	+++	-	+++	+++	+++
Met	+++	+++	+	+++	+	+++
Cys	+	+++	+	+++	+++	+++

- = absence of absorbancy
++ = absorbancy 0.10 - 0.18,
+++ = absorbancy > 0.19
aa[1] = pool of 17 amino acids.
* class - 1st - without any inhibitory effect
2nd - complete absence of growth,
3rd - some inhibitory effect in the bacterial growth.

bacterial growth. Probably changes in the broth composition occurred prior to the acid accumulation due to the cell recyclings in these situations.

The growth inhibition of *L. fermentum* observed in the control during the early cycles was not detected when yeast extract (Fig. 1) or pure amino acids were added to the broth (Fig. 2). A similar result was obtained in a previous work in which a significant decrease (14.4%) in alcoholic efficiency occurred when yeast extract was supplemented in the wort of alcoholic fermentation contaminated with *L. fermentum* (19).

Since the initial conditions of substrate were similar in all treatments, the difference in stimulation of bacterial growth could not be attributed to monosaccharides released by sucrose hydrolysis as claimed by Essia-Ngang *et al.* (6). Their work was conducted without cell recycling, and only the first cycle was analyzed. The nutritional changes must have happened in the following cycles, similarly to what was detected in our work.

The stimulation of bacterial growth when ethanol was present in a low concentration during yeast alcoholic fermentation was detected by King and Beelman (11). With the cell recycling process, the stimulation was detected only when yeast extract or a combination of amino acids was added to the broth. When these nutrients were omitted, a decrease in the number of bacteria during the first cycle was detected. This evidenced that the stimulation of bacterial growth was not due to ethanol, since the initial conditions were the same for control and treatments (amino acids and yeast extract).

The cell recycling process caused a medium enrichment since dead cells were recycled and concentrated in the broth. Yeast autolysis due to yeast proteinases (16) and protein hydrolysis carried out by peptidases present in lactobacilli (4) cause a release of amino acids and peptides in the fermented broth. About 30% of the nitrogen content is released by *Saccharomyces cerevisiae* cells due to autolysis, and amino acids represent 25% of these compounds (13).

Industrial alcoholic fermentation is run with a high concentration of yeast cells (10 - 15% v/v), and often with low yeast viability (40 - 60%) due to several process problems (7). So the release of a reasonable amount of amino acids and their concentration in the broth by cell recycling are expected. Yeast extract fractions showed that amino acids are responsible for the stimulation of lactic acid bacteria growth (22). Yeast extract was divided into fractions by ion

exchange chromatography. The separated amino acids Leu, Ile, Val, Ser, Glu, Asp, Thr, Gly, Ala, Tyr, Lys were free or linked to the yeast cell (20). The linked forms were probably returned with pitching cell suspensions in the cell recycling process of yeast alcoholic fermentation.

Genetic lesions are responsible for amino acid requirements in *Lactobacillus* (15). Absence of Leu, Ile, Val, Phe, Trp, Glu or Arg led to growth inhibition in *L. plantarum*, *L. casei*, *L. helveticus* and *L. acidophilus* (15). Leu, Ile or Val, when not introduced into the medium, caused a complete growth inhibition of *L. fermentum* (Table 4). In fact, the biosynthesis pathway common to these 3 essential amino acids seems not to exist or is inactive in lactic acid bacteria (15). Probably, several amino acids were essential to the growth of *L. fermentum*. All essential amino acids might have been present to a certain extent in the dscb. since the growth of *L. fermentum* occurred, although at a lower rate, even though one of the other possibly essential amino acids (ex. Phe, Trp, Glu, Tyr) was not introduced into the medium (Table 4). Leu, Ile and Val compete with the same carrier but at different rates and the unbalance in the concentration of these amino acids in the medium could make decrease or inhibit the growth of the cells by competitive uptake (3). For example, the Leu consumption in *L. casei* is entirely inhibited by excess of Val and Ile (23).

Since sugar cane juice is poor in proteins or amino acids and these products stimulate bacterial contaminants, it is desirable, in industrial practices, to maintain a high yeast viability and a low yeast concentration (to a reasonable extent), in order to reduce yeast extract in the wort, this way avoiding the stimulation of contaminant growth. Since Leu, Ile or Val are essential for the growth of *L. fermentum*, the elimination of these components in the wort by some technological means could be researched as a future method of contaminant control in industrial alcoholic fermentation with ecological advantages.

The main conclusions of this study are:

1 - The mixed culture with *S. cerevisiae* and *L. fermentum* confirms the competition of these microorganisms. At the beginning, bacteria suffer a growth inhibition, but after some cycles yeast growth is inhibited by the acidity due to the accelerated bacterial growth.

2 - The growth inhibition of *L. fermentum* during the first fermentation cycles is due to nutritional deficiencies. With cell recycling, nutrients from dead yeast are realeased into the medium stimulating the *L. fermentum* growth.

3 - The amino acids Leu, Ile and Val must be added to sugar cane broth to stimulate *L. fermentum* growth but Tyr, Asp or Lys are not essencial. Phe, Ala, Glu, Cys, Pro, His, Arg, Thr, Trp, Ser, Met influence in some degree the *L. fermentum* growth in the sugar cane broth .

ACKNOWLEDGEMENTS

The authors thank the Fundação Tropical de Pesquisas e Tecnologia André Tosello (Campinas, SP, Brasil) for the maintenance of the cultures and for providing some equipment for this work, and the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support.

RESUMO

Efeitos de fatores nutricionais no crescimento de *Lactobacillus fermentum* em cultivo misto com *Saccharomyces cerevisiae* na fermentação alcoólica.

O crescimento de *Lactobacillus fermentum* foi estudado em cultivo misto com *Saccharomyces cerevisiae* na fermentação alcoólica do HTM (high test molasses). Extrato de levedura ou um grupo de 17 aminoácidos causou forte e rápido decréscimo na viabilidade das leveduras devido ao aumento da acidez produzida pelas bactérias. Cultivo puro de *L. fermentum* no meio a base de caldo de cana-de-açúcar desidratado confirmou os aminoácidos como os principais nutrientes necessários para estimular o crescimento do contaminante bacteriano na fermentação alcoólica. Ausência do crescimento de *L. fermentum* foi obtida quando leucina, isoleucina ou valina não foram complementadas no meio. Fenilalanina, alanina, ácido glutâmico, cistina, prolina, histidina, arginina, treonina, triptofano, serina e metionina inibiram o crescimento bacteriano em pelo menos uma das culturas de *L. fermentum* testadas.

Palavras-chave: aminoácidos, estimulação, crescimento, *Lactobacillus fermentum*, *Saccharomyce cerevisiae*, cultivo misto.

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Revista de Microbiologia (1997) 28:32-39
ISSN 0001-3714

MICROBIAL AGENTS RELATED TO SEXUALLY TRANSMITTED DISEASES

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ABSTRACT

Urogenital specimens from 663 out and inpatients of the Pedro Ernesto Hospital of the State University at Rio de Janeiro (HUPE-UERJ) were examined for the presence of some relevant pathogens of sexually transmitted diseases (STD), as well as for other microorganisms. All pathogens were isolated after culture in specific media except *Chlamydia trachomatis* that was investigated by the direct immunofluorescence technique. *C. trachomatis, Candida albicans* and *Gardnerella vaginalis* occurred in 114 female patients (46.3%) and *C.trachomatis*, *Ureaplasma urealyticum* and *Neisseria gonorrhoeae* were detected in 115 male patients (27.6%). Association of two or more microrganisms was found in 71 (10.7%) occasions. These associations were more frequent in women (17.9%) than in men (6.2%). The prevalence of mycoplasmas (*Mycoplasma hominis* and *U. urealyticum*) was lower than the levels quoted in the literature. The isolation of *U. urealyticum* in semen was more than twice (19.8%) the one found in urethral secretions (9.2%) or in samples of first-voided urine (8.6%). The association between *M. hominis* and *G. vaginalis* ($p<0.05$), which is described as a result of unbalanced vaginal microbiota, was also observed. A systematic search for different potential agents of STD is emphasized, in view of the marked occurence of associations between the detected microorganisms.

Key words: Sexually Transmitted Diseases (STD), microbial associations, laboratorial diagnosis

INTRODUCTION

The sexually transmitted diseases (STD) constitute one of the greatest concerns of public health, specially considering the increasing incidence of various agents, some of which determine serious complications (5, 29). The great variety of microorganisms that are STD agents, such as protozoans, fungi, bacteria, chlamydiae and viruses, makes both clinical and laboratorial diagnosis difficult (29).

The importance of a restrict group of agents of veneral diseases with emphasis to *Neisseria gonorrhoeae* e *Treponema pallidum*, and also to *Haemophillus ducrey* and *Chlamydia granulomatis*, is historically recognized. Nowadays, the viruses of human immunodeficiency and the varieties of *Herpes simplex* are also recognized as agents of great importance.

Among the nonviral agents, much emphasis has been given to *C.trachomatis*, which is, undoubtedly, the most important agent of non-gonococcal urethritis and cervicitis and can determine serious complications in the male and female genital tracts (8, 24). In women, it mainly causes cervicitis, being the most prevalent

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agent in these cases, although it is not considered an agent of vaginitis (15). Assymptomatic urethritis, usually affecting men, contributes to the infection of women. Thus, the research of these agents is very important in high-risk groups (31). Cultures of endocervical specimens that become negative after the agents have progressed to the upper genital tract seems to be a great problem. Such situation has lead Arena *et al.*(2) to propose the research of the agent in the peritoneal liquid obtained by laparoscopy.

Related to the group of mycoplasmae (*M. hominis and U. urealyticum*), there are doubts about its phathogenicity, since it is frequently found as colonizer of the urogenital mucosa, and occurs in more than 50% of sexually active adults (34). *M. hominis* is commonly associated to the occurrence of vaginosis, but its role in urethrites is highly questionable (33). It can be responsible for the appearance of abscesses, puerperal infections, bacteremia, serious infections in patients with burn wounds, infections of the central nervous system in newborns and pelvic inflamatory disease (34).

U. urealyticum is a relevant agent of the nongonococcal urethritis. Based on the etiology, some authors consider it more significant in clinical cases than in controls (22, 28). It has not been considered an agent of vaginitis and cervicitis, but is commonly associated with spontaneous abortion, infertility, salpingitis, puerperal fever and prematurity. Hall *et al.* (15), however, observed that *U.urealyticum* was twice more frequent in cases of vaginitis than in assymptomatic patients.

More recently, *M. genitalium* has been recognized to be as relevant in nongonococcal urethritis as *U. urealyticum* (17).

Bacterial vaginosis is characterized by alteration of the normal vaginal microbiota, which is predominantly constituted by *Lactobacillus sp., Gardenerella vaginalis*, some species of *Mobiluncus* and the genital micoplasmae. Sexual activity is thought to be a predisposing cause of this type of infection. This cause is questionable and it does not exactly constitute a STD (23).

Ansel *et al.* (1) detected *G. vaginalis* in 40% of assymptomatic patients. In contrast, they observed a significant association of this microorganism with nonspecific vaginitis. In Brazil, Peixoto *et al.* (25) described 25.3% of prevalence of *G. vaginalis* in cases of nonspecific vaginitis, except for the cases compatible with infection by *Trichomonas vaginalis.*

Some authors accept the controversial proposal that complications of vaginosis can lead to upper genital infections (12). Some other authors, however, consider this a premature view, since vaginosis was not found in patients with pelvic inflammatory disease (14).

T. vaginalis is a recognized agent of vaginitis and urethritis (22). If changes in the vaginal microenvironment occurs, *Candida albicans* colonizes the vaginal epithelium, determining the appearance of vaginitis, frequently of recurrent character (30).

This investigation searched for different agents directly or indirectly related to infections of the male and female genital tract. We studied specimens from in and outpatients at the HUPE-UERJ, of different ages, during a period of four years.

MATERIALS AND METHODS

Population. Patients which were attended at HUPE-UERJ from July 1987 up to August 1991, with clinical complaints, suggestive of STD and mostly with no treatment, were studied. A few had received some kind of therapy without medical orientation, and presented post-therapy recurrence. The female patients were from outpatient services and/or from gynecology, obstetrics and prenatal clinics. Most male patients were from outpatient services, while the majority of the inpatients were from the urological clinic. Teenagers, both male and female, were from the adolescent clinic of HUPE-UERJ. A few cases included patients under therapeutic control. The study included 663 patients, being 246 females (243 specimens of uterine colon and 3 urethral secretions) and 417 males (229 urethral secretions, 101 semen samples, 81 samples of first-voided urine, 1 prostatic and 6 glans secretions). Endocervical secretions, from female patients, for research of *C. trachomatis* were obtained. Among the 663 patients, 89 were teenagers and children (26 males and 63 females).

Collection of specimens. The patients were requested to go to the laboratory before the midstream early morning urine and to produce a control specimen at least four days after interruption of the treatment (22). The male secretions were obtained in the laboratory with a sterile swab of thin wire, introduced 2 to 4 cm in the urethra and slightly scrubbed against the mucosa. For the collection of female specimens, the physicians were oriented to take them from the uterine colon with a common sterile swab, using a speculum, aiming the culture procedures, and to obtain the endocervical specimens, with a sterile thin swab

for research of *C. trachomatis*. In few cases, other clinical materials such as female urethral, glans and prostatic secretions, were obtained.

Culture and Isolation of Microorganisms. For the isolation of *N. gonorrhoeae*, Thayer Martin medium was used (26). Eighteen hours after incubation at 37°C, in 5-10% CO_2 atmosphere, the suspected colonies were examined by Gram smears and were submitted to oxidase and other biochemical tests (fermentation of glucose, maltose and sucrose). *M. hominis* was isolated on A7 differential medium (28) and incubation in CO_2 atmosphere for 48 hours at 37°C followed by direct microscopic (40 X) observation of the colonies. Diamond Broth supplemented with 1000 units/ml of penicilin, 500 mg/ml of streptomicin and 50 mg/ml of nistatine was used for detection of *T. vaginalis* (32). The medium was incubated at 37°C for a period of 4 days and the search for the parasite was done in hanging-drop preparations. For detection of *U. urealyticum*, liquid medium U9 incubated for 48-72 hours at 36°C was used. Reading was based on the observation of the changing shift of the medium from yellow to red (28).

To isolate *C. albicans* Biggy Agar was used and the cultures were incubated at 37°C for 48 hours. Suspected colonies were submitted to microscopy and to "germ tube" test (10). For isolation of *G. vaginalis*, Columbia Agar supplemented with 3% human blood was used. After incubation under microaerophilic conditions at 37°C for 48 hours, suspected colonies were submitted to Gram stain and tested for catalase, hemolysis and sensitivity to 5% sodium polianetosulfonade (SPS). All specimens were also inoculated in Columbia Agar supplemented with sheep blood, in 5% Chocolate Agar and in Polyvitex (bio Mérieux).

Direct immunofluorescence microscopy, using monoclonal antibodies (Difco or bio-Mérieux), was used for the detection of *C. trachomatis*.

Catalase positive Gram-positive cocci isolated in blood agar and/or chocolate agar were submitted to coagulase test, fermentation of mannitol and DNAse production (18). The catalase negative β-hemolytic streptococci were submitted to sensitivity tests using discs (Cecon), to CAMP Test (13) and also serogrouped by latex agglutination using Strepto kit ABCDFG (bio Mérieux). The α-hemolytic streptococci were tested for susceptibility to optoquin (Cecon) and to 6,5% NaCl. Oxidase-negative glucose-fermentative gram negative rods were identified by biochemical tests for *Enterobacteriaceae* (11). The non fermentative gram negative rods were identified by biochemical tests according to Rubin *et al.* (27).

For analysis of results, positive cases were the ones in which one or more of the following microorganisms were isolated: *C. trachomatis, G. vaginalis, C. albicans, U. urealyticum, M. hominis, T. vaginalis, N. gonorrhoeae,* and "other microorganisms" like coagulase positive or negative *Staphylococcus, Streptococcus sp., Enterobacteriaceae* and non fermentative Gram negative rods, etc. Negative cases were those in which no microorganism was detected in culture and were negative for *C. trachomatis* by direct immunofluorescence.

A summary of cultural methods is presented in Table 1.

Statistics. The chi-square test, applied according to Berquó *et al.* (4), was used for the comparative analysis of results concerning frequency of the microorganisms that were isolated and the associations between them.

Table 1 - Summary of the culture media, conditions of incubation and results of the different microorganisms researched.

Microorganisms	Media	Incubation	Results
Ureaplasma urealyticum	U9	72 h	+ pinkish limpid medium - yellowish medium
Trichomonas vaginalis	Diamond	5 days	wet preparations (40 X) examined on 3rd and 5th days
Mycoplasma hominis	A7	anaerobiosis	colonies examined with inverted microscope
Neisseria gonorrhoeae	Thayer Martin	Low O_2 tension (48 h)	Gram, oxidase and sugar fermentation tests
Candida albicans	Biggy Agar	48 h	Gram, "germ tube", and colonial morphology
Gardnerella vaginalis	3% human blood Agar	Low O_2 tension (48 h)	Pleomorfic Gram negative rods, catalase, hemolysis, sensitivity to 5%SPS

RESULTS

Among 246 specimens from female patients, 141 were considered positive. From these, 192 relevant microorganisms were obtained. In 81 patients, other microorganisms were isolated. Specimens from 24 patients were negative (Table 2). *G. vaginalis, C. trachomatis and C. albicans* were most frequent microorganisms and they were detected in 114 (46.3%) of the female positive samples.

From 417 specimens from male patients, 175 were positive for significant germs. However, in 198 of the patients, the microorganisms that were isolated probably did not have influence in the infectious process. *C. trachomatis, N. gonorrhoeae* and *U. urealyticum* were isolated in 115 (27.6%) of the male patients (Table 3). The frequency of *U. urealyticum* in samples of semen (19.8%) was twice the one found in first-voided urine (8.6%) and also in urethral secretion (9.2%). *M. hominis* showed similar frequencies: 4.0%, 5.0% and 6.5%, respectively (Table 3). *G. vaginalis* was detected in 12 (2.9%) and *C. albicans* in 4 (< 1%) of the male specimens.

No growth was obtained in specimens of 24 female patients (9.7%) and of 73 male patients (17.5%), corresponding to 97 (14.3%) of the total cases examined. In 279 cases (42.1%), growth of microorganisms other than those considered relevant was observed. From these, 81 (32.9%) were from female patients and 198 (47.5%) from male patients (Tables 2 and 3). Most of these microorganisms correspond to commensals, considered normal constituents (permanent or not) of the microbiota associated with the male and female urogenital tract, such as α-hemolytic streptococci, gram positive bacilli, nonidentified aerobic bacteria, coagulase negative staphylococci, nonfermentative gram negative rods and *Enterobacteriaceae* (more frequently *E. coli*). In few cases, *S. aureus* and β-hemolytic *Streptococcus* of groups A and B were isolated.

The subgroup of adolescents were examined separately. There was no difference regarding the agents that were isolated and their frequency. Nevertheless, the frequency of isolation of *N. gonorrhoeae* was higher in male adolescents than in male adults. In female adolescents, the frequency of isolation of *M. hominis* was lower compared to female adults.

Considering the total number of patients, association of two or more species of relevant microorganisms was detected in 70 (24.3%) out of 288

Table 2 - Prevalence of microorganisms in genital specimens of female patients of HUPE-UERJ

	PATIENTS[1] - *n (%)*									
	C.trachom[2]	*C. alb*[3]	*T. vag*	*G. vag*	*N. gon*	*M. hom*	*U. urea*	Other	Neg	Total
Cervical	40 (16.5)	41 (16.9)	9 (3.7)	52 (21.4)	2 (<1)	12 (5.0)	32 (13.0)	81 (33.3)	24 (9.9)	243 (100)
Urethral	1 (33.3)	1 (33.3)	1 (33.3)	0	0	1 (33.3)	0	0	0	3 (100)
Total	41 (16.7)	42 (17.1)	9 (3.6)	53 (21.5)	2 (<1)	13 (5.3)	32 (13.0)	81 (32.9)	24 (9.7)	246 (100)

(1) some cases presented more than one agent.
(2) the clinical material used in the detection of *C. trachomatis* by direct immunofluorescence (microscopy) was endocervical secretion.
(3) C.alb = *Candida albicans*; N.gon = *Neisseria gonorrhoeae*; T. vag = *Trichomonas vaginalis;* M.hom = *Mycoplasma hominis;* U.urea = *Ureaplasma urealyticum*; other = other microorganisms like *Enterobacteriaceae, Staphylococcus sp., Streptococcus sp.* etc.; Neg = absence of microbial growth.

Table 3 - Prevalence of microorganisms in genital specimens in male patients of HUPE-UERJ

	PATIENTS[1] - *n (%)*									
	C.trachom[2]	*C. alb*[3]	*T. vag*	*G. vag*	*N. gon*	*M. hom*	*U. urea*	Other	Neg	Total
Urethral	35 (15.3)	1 (<1)	2 (<1)	4 (1.7)	29 (12.7)	15 (6.5)	21 (9.2)	111(48.5)	31(13.5)	229(100)
semen	8 (7.9)	3 (3.0)	0	5 (4.9)	0	4 (4.0)	20 (19.8)	44 (43.6)	21(21.8)	101(100)
first-voided urine	8 (10.0)	0	0	3 (3.7)	1 (<1)	4 (5.0)	7 (8.6)	42 (51.8)	21(25.9)	81 (100)
Glans lesion	3 (50.0)	0	0	0	0	1 (16.7)	1 (16.7)	1 (16.7)	0	6 (100)
Total	54 (12.9)	4 (<1)	2 (<1)	12 (2.9)	30 (7.2)	24 (5.7)	49 (11.7)	198 (47.5)	73(17.5)	417(100)

(1) some cases presented more than one agent.
(2) see foot note 3 in Table 2.

Table 4 - Prevalence of relevant microorganisms exclusively or in association with other germs.

Microorganisms	Positive patients - number (%)					
	Female			Male		
	exclusive	associated	total	exclusive	associated	total
G. vaginalis	28 (19.8)	25 (17.7)	53 (37.6)	7 (4.8)	5 (3.4)	12 (8.2)
C.trachomatis	24 (17.0)	17 (12.0)	41 (29.1)	42 (28.6)	12 (8.2)	54 (36.7)
C. albicans	21 (14.9)	21 (14.9)	42 (29.8)	3 (2.0)	1 (0.7)	4 (2.7)
U. urealyticum	18 (12.8)	14 (9.9)	32 (22.7)	30 (20.4)	19 (12.9)	49 (33.3)
T. vaginalis	3 (2.1)	6 (4.2)	9 (6.4)	1 (0.7)	1 (0.7)	2 (1.4)
M. hominis	3 (2.1)	10 (7.1)	13 (9.2)	15 (10.2)	9 (6.1)	24 (16.3)
N. gonorrhoeae	0	2 (1.4)	2 (1.4)	22 (15.0)	8 (5.4)	30 (20.4)
TOTAL	97 (68.8)	44 (31.2)	141(100)	120(81.6)	26 (17.8)	140(100)

Table 5 - Caracteristics of the associations of microorganisms in positive female patients

	Associated germs[1]							
	Chlam	Cand	Trich	Gard	Neiss	Mycop	Ureap	Total[2]
Chlam	24 (58.5)	6 (14.6)	2 (4.9)	4 (9.7)	2 (4.9)	2 (4.9)	7 (17.1)	41 (100)
Cand	6 (14.3)	21 (50.0)	3 (7.1)	11 (26.2)		1 (2.4)	2 (4.8)	42 (100)
Trich	2 (22.2)	3 (33.3)	3 (33.3)	1 (11.1)			4 (44.4)	9 (100)
Gard	4 (7.5)	11 (20.7)	1 (1.9)	28 (52.8)		7 (13.2)	6 (11.3)	53 (100)
Neiss	2 (100)							2 (100)
Micop	2 (15.4)	1 (7.7)		7 (53.8)[3]		3 (23.1)	2 (15.4)	13 (100)
Ureap	7 (21.9)	2 (6.2)	4 (12.5)	6 (18.7)		2 (6.2)	18 (56.2)	32 (100)

Chlam = *C. trachomatis*; Can = *C. albicans*; Trich = *T. vaginalis*; Gard = *G. vaginalis*; Neiss = *N. gonorrhoeae*; Mycop = *M. hominis*; Ureap = *U. urealyticum*

1 = occurrence of association of two or more germs

2 = number of individuals presenting the germ , excluding the repetitions in the counting of the associations

3 = statistically significant associations related to the presence of *G. vaginalis* and *M. hominis* ($p<0.05$)

positive cases. The prevalence of these associations in positive cases was higher in female (31.2%) than in male patients (17,8%) (Table 4). The analysis of the different associations that were observed did not show probable dependencies, except for *G. vaginalis* and *M. hominis* among female patients ($p< 0.05$). For most of the expected associations, the frequencies were not enough to permit the application of the statistical test.

Regarding female patients, *T. vaginalis* and *M. hominis* occurred most times in associations (Table 5), while in male patients, the frequencies of single microorganisms were higher than the associations (Table 6).

DISCUSSION

The data obtained in this study were comparable to those reported in the literature. In female patients, a greater prevalence of vaginosis and candidiasis was detected. However, the frequency of isolation of *G. vaginalis* may lead to an overestimation of vaginosis, since the higher frequency of this agent is not enough to afford a real characterization of the clinical presentation. In terms of frequency of isolation, *C. trachomatis* appears as the third agent, confirming its importance in clinical presentations of cervicitis, although cervicitis probably corresponds to only a part of the female genital infections.

The high frequency of association of germs shows the importance of a wider search for potential agents. Sobel (30) reports that, although two or more agents are usually isolated in 15 to 20% of the cases of vaginitis, broader epidemiological investigations are needed, specially because there are several difficulties in the detection of certain individual agents, such as *U. urealyticum* and *M. hominis,* when more complex culture media are used. For *C. trachomatis*, technical

Table 6 - Caracteristics of the associations of microorganisms in positive male patients

	Associated germs[1]							
	Chlam	Cand	Trich	Gard	Neiss	Mycop	Ureap	Total[2]
Chlam	42 (77.8)		1 (1.8)	1 (1.8)	3 (5.5)	2 (3.7)	10 (18.1)	54 (100)
Cand		3 (75.0)					1 (25.0)	4 (100)
Trich	1 (50.0)		1 (50.0)				1 (50.0)	2 (100)
Gard	1 (8.3)			7 (58.3)		4 (33.3)	2 (16.7)	12 (100)
Neiss	3 (10.0)				22 (73.3)	1 (3.3)	5 (16.7)	30 (100)
Micop	2 (8.3)	1 (7.7)		4 (16.7)	1 (4.2)	15 (62.5)	5 (20.8)	24 (100)
Ureap	10 (20.4)	1 (2.0)	1 (2.0)	2 (4.1)	5 (10.2)	5 (10.2)	30 (61.2)	49 (100)

Chlam = *C. trachomatis;* Cand = *C. albicans;* Trich = *T. vaginalis;* Gard = *G. vaginalis*; Neiss = *N. gonorrhoeae*; Mycop = *M. hominis;* Ureap = *U. urealyticum*

1 = occurrence of associations of two or more germs

2 = total individuals presenting the germ, excluding the repetitions in the counting of the associations

3 = statistically significant associations related to the presence of *G. vaginalis* and *M.hominis* ($p<0.05$)

ability for the use of the direct immunofluorescence method is required for a correct microscopic reading.

Keeping in mind that sensitivity of immunoenzymatic tests may be insufficient to detect *C. trachomatis* in first-voided urine of patients with urethritis (3), much is being done in order to obtain a more effective system for the detection of this agent (19). The utilization of direct immunofluorescence plays an important role in routine diagnosis, since results are comparable to those obtained in the detection of this agent by polymerase chain reaction ("PCR") (35).

Microscopy may be an important laboratorial resource and it can be broadely used, specially in the routine investigation of vaginosis (36). Moreover, the detection of "clue cells" is also useful because once positive, culture is not needed (7), nor is the evaluation of the *Lactobacillus* morphotypes (16). The present findings point out the reduced participation of *T. vaginalis* in female genital infections.

In male patients, the frequency of isolation of *U. urealyticum* and *C. trachomatis* was comparable. In fact, the important role of *U.urealyticum* agent in the nongonococcal urethritis is being emphasized, mainly when associated with therapy failure (5).

Many authors believe that the presence of *G. vaginalis* in male partners is not relevant to the development of vaginosis (23). Nevertheless, this microorganism was detected in 12 (2,9%) male patients. The potential role of reservoirs of sexually transmitted agents, specially in cases of therapeutic failure, was referred (21). The finding of *C. albicans* in men (less than 1% in this study) lead many authors to consider the possible existence of reservoirs of this agent as responsible for recurrent infections in women. However, this possibility has been questioned (30).

With regard to the isolation of *C. trachomatis*, the frequency in which the complications presumably appeared, shows a tendency of the cervical cultures to become negative so that collection of specimens from other sites can be proposed (2). This would underestimate the prevalence of this agent.

The importance of quantification of genital mycoplasmas has been emphasized, considering that they frequently are associated with the microbiota of the urogenital tract. Legris (21) points out the need of a sorological evaluation in order to differenciate infection and colonization. This was not the aim of the present study and only the prevalence of the microorganisms was determined. The frequency of mycoplasmas (*M.hominis and U. urealyticum*) was low, which contrasts with other reports (9, 33). Factors like collection conditions, incubation periods or variations in the population studied, isolated or in associations, would explain the this low frequency. On the other hand, the more specific analysis of semen samples, in comparison with urethral secretion and first-voided urine, showed that the frequency of *M. hominis* in these samples was similar, while *U. urealyticum* were twice more frequent in semen. This emphasizes the need for the search of the microorganisms in cases of urethro-prostatitis, since they have been recognized as potential agents of this type of infection (9).

In general, *M. hominis* and *U. urealyticum* were not more frequent when associated with other microorganisms as compared with a single isolation. However, in female patients the association of *M.*

hominis with *G. vaginalis* was significant ($p<0.05$), which is an interesting fact in vaginosis (14).

Other agents, which were isolated but not individually described, seem to be associated to infectious processes, specially vaginitis, in patients under the effects of antimicrobials or immunodepressed. The exacerbation of the normal microbiota could be one of the factors responsible for clinical vaginitis (20). As a routine, the physicians were informed of the results of such findings, specially in cases where a single significant microorganism was isolated. In these cases, antimicrobial susceptibility tests were done.

Considering the complexity of the great variety of agents of STD, the ample analysis of these agents gives support to the clinical decision, since the role of the microorganism is not very clear. It also contributes to the quality control of the routine method, since a significant frequency of agents in associations is expected and these agents must be detected in determined confidence intervals.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Marcelo Magalhães, Dr. Marlene Andrade, Dr. Adelma Véras and Dr. Raimundo Diogo Machado for technical orientation, Dr. Nelson Jerônymo Lourenço, Dr. Eduardo Ribeiro de Castro and Dr. Mara Lucia Penna Queiroz for their suggestions and M. Neuma Gloria Duarte Pereira for the translation into English.

We also acknowledge CNPq and Laboratório Zambon for their support.

RESUMO

Agentes microbianos relacionados com doenças sexualmente transmitíveis

Foram estudados espécimes clínicos de 663 pacientes atendidos nos ambulatórios ou internados em clínicas do HUPE-UERJ, apresentando sintomatologia compatível com quadros de infecções genitais. Para todos foi pesquisada a presença de alguns patógenos relevantes em doenças sexualmente transmissíveis (DST) além de outros germes. *C.trachomatis* foi pesquisada por técnica de imunofluorescência direta. Os demais germes foram isolados a partir de diferentes espécimes clínicos em meios de cultura específicos. Verificou-se que *C.trachomatis*, *C.albicans* e *G.vaginalis* constituiram-se nos microrganismos mais frequentemente detectados em 114 pacientes femininos (46,3%). *C.trachomatis*, *U.urealyticum* e *N.gonorrhoeae* foram detectados em 115 dos pacientes masculinos (27,6%). Em 71 (10,6%) do total de casos foram encontradas associações de dois ou mais microrganismos no mesmo paciente, mais frequentemente em mulheres (17,9%) do que em homens (6,2%).

As prevalências de micoplasmas (*M.hominis* e *U.urealyticum*) foram baixas em relação ao descrito na literatura. Entretanto, foi verificado que a frequência de isolamento de *U.urealyticum* em amostras de semen (19,8%) foi mais do que duas vezes maior do que em secreções uretrais (9,2%) ou amostras de urina de 1° jato (8,6%). Foi também constatada a associação entre *M.hominis* e *G.vaginalis* ($p<0.05$), que é descrita como decorrente do desequilíbrio de microbiota vaginal.

Enfatiza-se a necessidade de pesquisa sistemática de diferentes agentes potenciais de infecções genitais, discutindo as dificuldades envolvidas nesta tarefa, tendo em vista a marcante ocorrência de associações de microrganismos detectados.

Palavras-chave: doenças sexualmente transmitidas, associações microbianas, diagnóstico laboratorial

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Revista de Microbiologia (1997) 28:40-45
ISSN 0001-3714

PLASMID PROFILES OF *YERSINIA PESTIS* STRAINS ISOLATED IN NORTHEAST BRAZIL

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ABSTRACT

Plasmid composition in 182 *Y. pestis* strains from six natural plague foci of Northeast Brazil was analyzed by gel electrophoresis. One hundred and six strains (61.5%) displayed the classical plasmid pattern composed of the three well-characterized *Y. pestis* plasmids, fifty-six strains (33.3%) missed at least one of them and twenty (11.0%) strains displayed additional cryptic plasmids. These variations on the plasmid profile were observed among strains originated from the foci of Chapada do Araripe, Planalto da Borborema, Serra da Ibiapaba and Bahia while all the strains tested from the Triunfo and Serra de Baturité foci displayed the classical plasmide profile.

Key words: *Y. pestis*, plasmids, plague.

INTRODUCTION

Based on the study of a few laboratory strains (6, 9, 14) it was postulated that *Y. pestis*, the causative agent of plague, has a typical plasmid profile (pYV ≅ 70kb, pPst ≅ 9.5kb and pFra ≅ 90kb) 6, 9, 14, 16.

The pYV codes for calcium-dependent growth of cells incubated at 37°C and synthesis of a set of proteins called Yops (9, 15). pPst codes for pesticin as well as the production of a plasminogen activator and a coagulase (18). Finally, pFra codes for the F1 capsular antigen and the murine toxin (16).

Screening of the plasmid profiles in bacteria proved to be a rather simple and fast technique able to offer useful epidemiological data (13). Attempts to characterize the plasmid content of wild type *Y. pestis* strains have been restricted to samples isolated in Asia, mainly in the territory of the formerly called URSS (10) and in Mongolia (4). Most of the strains have shown to carry the three classical plasmids. However, it was observed that strains isolated from voles, rodents obtained in the Caucasus, lack the pPst plasmid. A few additional cryptic plasmids as well as a considerable size variation of the pFra and pYV plasmids have been observed, and a possible correlation with the geographical characteristics of these strains has been put forward (4, 10).

In Brazil, there are several independent *Y. pestis* foci in rural areas, mainly in the Northeast region (5, 20). A preliminary evaluation of the plasmid profiles of 26 strains, isolated during a plague outbreak at one of these foci (2) has revealed a homogeneous pattern composed of the three ubiquitous *Y. pestis* plasmids and an additional 22.5 kb extra chromosomal DNA band not recoverable by plasmid isolation techniques based on alkaline lysis performed in small scale extraction (11, 12).

In our present work we analyzed the plasmid content in 182 *Y. pestis* strains isolated from six plague foci in Northeast Brazil (1,2). The results of this study confirm the presence of the three classical *Y. pestis* plasmids in most of the Brazilian strains. Surprisingly,

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a fraction of the tested strains missed at least one of these plasmids whereas a few strains carried additional cryptic bands. These variations on the plasmid profile had been found among strains from the foci of Chapada do Araripe, Planalto da Borborema, Serra da Ibiapaba and Bahia. All the strains tested from the Triunfo and Serra de Baturité foci displayed the classical plasmide profile. No clear relationship could be drawn between plasmid profiles and geographical or epizootiological characteristics of the strains analysed.

MATERIALS AND METHODS

The 182 *Y. pestis* strains studied were from the bacterial strain collection of the "Centro de Pesquisas Aggeu Magalhães". These strains had been isolated from different hosts and distinct geographic foci from the Northeast Brazil during the period between 1966 to 1986 (1,2) and maintained at +4°C in stabs of Blood Agar Base (BAB, Difco Laboratories, Michigan USA). The reference strain *Escherichia coli* 39R861 from the National Collection Institute of Public Health Laboratory (Collin Dale, London) the vaccine strain *Y. pestis* EV 76 or our *Y. pestis* PPB 862 previously studied (2, 11, 12) were employed as controls. Before plasmid examination the strains were cultivated at 28°C in Brain Heart Infusion Broth (BHI = Difco) during 24 up to 72 h, plated on BAB plates to ensure purity, and grown for 24 h in BHI for plasmid extraction.

Plasmid extraction was performed by a small scale alkaline lysis technique based on the procedure described by Birnboim and Doly (7) followed by electrophoresis on 0,6% agarose gels and ethidium bromide staining.

The phenotypic plasmid markers calcium-dependent growth at 37°C coded by the pYV (9), synthesis of the bacteriocin pesticin coded by the pPst (18), and the synthesis of the F1 antigen coded by the pFra (16) were studied as described previously (3).

RESULTS

Plasmid analyses were carried out in 182 *Y. pestis* strains recovered from patients (76 strains), rodents (68 strains) and flea vectors (38 strains) from the plague foci of Chapada do Araripe (126 strains), Planalto da Borborema (3 strains), Triunfo (15 strains), Serra de Baturité (6 strains), Serra da Ibiapaba (29 strains) and Bahia (3 strains). Out of these, 113 strains (62.08%) displayed the classical plasmid profile composed by the pYV, pPst and pFra plasmids (Table 1).

Fifty-six strains (33.3%) missed at least one of these plasmids (incomplete classical plasmid profile) (Table 1). Among them, 46 strains missed 1 plasmid, 9 strains missed 2 and 1 strain missed all the three. Between these 56 strains, 22 (12,08%) lacked pPst (pPst$^-$ strains), 4 strains (2.19%) lacked pYV (pYV$^-$ strains) and 40 (21.97%) strains lacked pFra (pFra$^-$ strains). The pFra was the most frequently missing

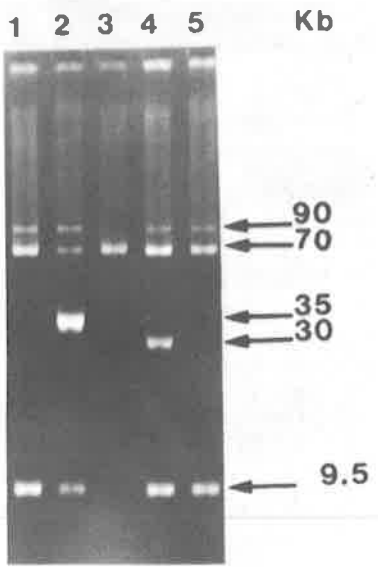

Figure 1: Plasmid profiles of some representative *Y. pestis* strains from different plague foci in Northeast Brazil. Lines 1, P.EXU 315; 2, P.EXU 216; 3, P.EXU 189; 4, P.EXU 115; 5, EV76 (vaccinal *Y. pestis* strain).

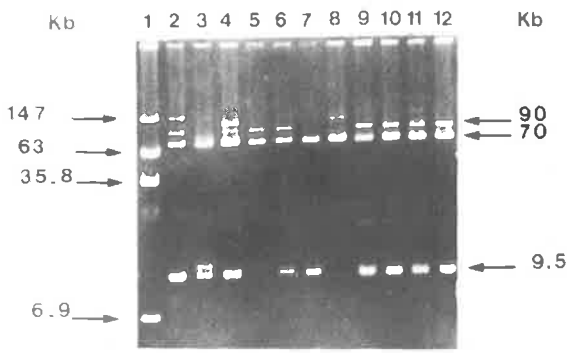

Figure 2: Plasmid profiles of some representative *Y. pestis* strains from different plague foci in Northeast Brazil. Lines 1, *E.coli* 39R861 (reference strain); 2, P.EXU 260; 3, P.EXU 264; 4, P.EXU 266; 5, P.EXU 267; 6, P.EXU 270; 7, P.EXU 271; 8, P.EXU 289; 9, P. EXU 275; 10, P.EXU 277; 11, P.EXU 420; 12, EV76 (vaccinal *Y. pestis* strain).

Table 1: Plasmid profile of the brazilian *Yersinia pestis* strains.

Origin of the strains			Plasmid Profile			
Foci	Year	Nº strains	A	B	C	D
Chapada do Araripe	1966-1975	126	73	08	40	05
Ibiapaba	1971-1982	29	16	04	07	02
Planalto da Borborema	1979-1986	03	01	-	02	-
Triunfo	1967-1979	15	15	-	-	-
Bahia	1978	03	02	01	-	-
Baturité	1978	06	06	-	-	-
Total		182	113	13	49	07

A = classical plasmid profile
B = classical plasmid profile with additional cryptic bands
C = incomplete classical plasmid profile
D = incomplete classical plasmid profile with additional cryptic bands

Table 2: *Yersinia pestis* strains cured of at least one of the virulence-associated plasmids

Plague foci	Nº Strains	Cured plasmid[a]			Phenotipic expression[b]		
		pFra	pYV	pPst	$F1^+$	Cad^-	Pst^-
Chapada do Araripe	45	31	2	17	29	2	17
Serra da Ibiapaba	9	7	2	4	7	2	4
Planalto da Borborema	2	2	-	1	2	-	1

a = number of strains in which each plasmid was missing
b = +, expression of the plasmid-encoded phenotype; -, no detectable expression of plasmid-encoded phenotype
$F1^+$ = F1 production.
Cad^- = No detectable expression of calcium dependence at 37°C.
Pst^- = No detectable expression of Pesticin production.

plasmid (40/56), followed by the pPst (22/56) and pYV (4/56). These results could suggest that plasmid pYV is more stable than the other two plasmids. Plasmid-lacking strains were isolated from the foci of Chapada do Araripe (45/126), Serra da Ibiapaba (9/29) and Planalto da Borborema (2/3) while all the strains from Triunfo, Serra de Baturité and Bahia had all the three classical plasmids (Table 2).

The analyses of the phenotypic expression of some prominent plasmid-encoded properties have shown that accordingly all the 22 $pPst^-$ strains were negative for the expression of pesticin (18), the 4 pYV^- strains were calcium independent as they were able to grow at 37°C in the MOX medium (14). Curiously enough, 38 $pFra^-$ strains remained proficient for the F1 antigen synthesis whereas only 2 were unable to do so (Table 2).

Twenty strains (11.0%) carrying additional cryptic bands with molecular weights (147 to 11.5 kb), distinct from those of the classical plasmids, were found (Table 3; Figs. 1, 2). Among these strains, 13 had all the typical plasmids (classical plasmid profile with additional cryptic bands) while 7 have also shown the lack of at least one of the classical *Y. pestis* plasmids. (incomplete classical plasmid profile with plasmid additional cryptic bands) (Table 3 and Fig. 1). *Y. pestis* strains carrying cryptic bands were found in only three plague foci: 13 out of 126 strains (10.3%) from the Chapada do Araripe focus, 6 out of 29 (26.7%) from the Serra da Ibiapaba focus and 1 out of 3 (33.3%) from the Bahia focus. Additional bands could be detected in strains isolated from men, rodents and fleas regardless of the isolation period (Table 3).

Based on the molecular weight, the additional cryptic bands could be grouped into three classes: one composed of bands greater than the pFra, another group with sizes ranging between those corresponding to pFra and pYV and one group smaller than the pYV but greater than the pPst (Figs. 1, 2; Table 3).

In conclusion, four major plasmid patterns can be distinguished for the brazilian *Y. pestis* strains (Table 1). Most of them (62.0%) displayed the classical plasmid profile. The others (38%), can be distributed into three different patterns: incomplete classical plasmid profile, caracterized by the absence of at least one of the typical plasmids (26.9%); classical plasmid

Table 3: The 20 *Y.pestis* strains carrying additional cryptic plasmids, their phenotypes, hosts, geographical origin and year of isolation.

Strains	Plasmids (kb)								F1	Cad	Pst	Hosts	Foci	Year
P.EXU 260	>90	90		70				9.5	+	+	+	Man	C. Araripe	1968
P.EXU 861	>90	90		70				9.5	+	+	+	Rodent	S. Ibiapaba	1982
P.EXU 266	>90	90		70				9.5	+	+	+	Man	C. Araripe	1968
P.EXU 420	>90	90		70				9.5	+	+	+	Flea	C. Araripe	1970
P.EXU 274	>90			70					+	+	-	Man	C. Araripe	1968
P.EXU 289	>90			70				9.5	+	+	-	Man	C. Araripe	1968
P.EXU 114	>90	90		70				9.5	+	+	+	Rodent	C. Araripe	1967
P.EXU 273		90	82	70				9.5	+	+	+	Rodent	C. Araripe	1968
P.EXU 263		90	82	70				9.5	+	+	+	Rodent	C. Araripe	1968
P.EXU 799		90	82	70				9.5	+	+	+	Rodent	Bahia	1978
P.EXU 554		90	82	70				9.5	+	+	+	Man	S. Ibiapaba	1972
P.EXU 803		90	82	70					+	+	+	Man	S. Ibiapaba	1978
P.EXU 556		90	82	70				9.5	+	+	-	Man	S. Ibiapaba	1972
P.EXU 264			82	70			11.5	9.5	+	+	+	Man	C. Araripe	1968
P.EXU 429	>90			70				9.5	+	+	+	Flea	C. Araripe	1971
P.EXU 509			82	70				9.5	+	+	+	Man	S. Ibiapaba	1971
P.EXU 115		90		70		30		9.5	+	+	+	Flea	C. Araripe	1967
P.EXU 789		90		70	32			9.5	+	+	+	Man	S. Ibiapaba	1978
P.EXU 216		90		70	35				+	+	+	Rodent	C. Araripe	1968
P.EXU 228		90				29			+	-	-	Rodent	C. Araripe	1968

F1 = F1 production;Cad = Calcium dependence at 37°C;Pst = Pesticin production

profile with additional cryptic bands (7.1%) and incomplete classical plasmid profile with additional cryptic bands (3.8%).

DISCUSSION

The growing information on the plasmid content of wild strains of *Y. pestis*, originated from different natural plague foci in Asia, has allowed to relate distinct plasmid patterns to specific hosts or geographic origins of the strains (4, 10).

Our present work conducted on 182 *Y. pestis* strains recovered from diverse hosts and plague foci from Northeast Brazil has revealed that most of the strains displayed a classical plasmid profile composed of the three well-characterized plasmids: pYV, pPst and pFra. This might reflect the origin of the plague in Brazil which was introduced through a single entry site, the port of Santos in the Southeast region of the country, during the last great pandemic of plague at the end of the 19th century (20). The fact that a single strain has colonized the region and the relatively short period since then could explain this prominent plasmid pattern.

Strains lacking one, two or all the three classical *Y. pestis* plasmids have been observed. However, no correlation could be established between these strains and their origins. We do not believe that these strains could represent true wild type spontaneous variants since most of them were collected from diseased mammalian hosts, and essential virulence-associated factors in *Y. pestis* are plasmid encoded (8). Therefore, it is quite probable that some of these variants have been selected during the prolonged storage (up to 25 years). On the other hand, insertion sequences promoting the integration of the plasmids pYV and pFra into the chromosome of *Y. pestis* have been previously detected (17, 21). Therefore, it is also reasonable to speculate that the absence of plasmids in some strains represents their integration into the chromosome of the bacteria. Furthermore, the occurrence of 38 $pFra^-$ strains synthesizing F1 strengthens this hypothesis and is in agreement with previous observations that the pFra can integrate into

the *Y. pestis* chromosome without losing plasmid-encoded functions (17). A better evaluation of the integration of plasmids into the chromosome of the bacteria would demand the use of specific probes in Southern-blot experiments.

Another observation was the presence of additional cryptic extra chromosomal DNA bands of varied molecular mass on 20 strains originated from three foci. These findings confirm previous observation that the presence of additional plasmids in *Y. pestis* is a rather frequent phenomena (10, 19). Five different plasmids with molecular mass ranging from 35 to 11.5 kb were found respectively in each one of the five strains originated from two plague foci. Even though we could not establish any relevant epidemiological feature associated with these additional plasmids, they were remarkable for their stability on the respective strains, high copy number and their similarity with previously observed plasmids in wild (10) and laboratory *Y. pestis* strains (17). By contrast, the additional high molecular weight bands (a group higher than the pFra, and the other between the pFra and pYV) found in 16 strains are quite unstable since they can disappear by handling of the strains. Furthermore, the bacteria population in the strains carrying these high molecular weight bands is heterogeneous as such bands could not be recovered from all colonies obtained from a single plate (to be published elsewhere). These bands could represent an increase on the molecular mass of the pFra or the pYV, but as the pYV was nearly always present in the strains carrying extra bands and the pFra was present in 5 out of 8 strains harbouring higher bands this hypothesis is unlikely. On the other hand, it may be possible that these bands could represent an artifact generated by handling the samples during the plasmid extraction, open circular or linearised forms of one of the classical plasmids present in the same strain. Clear demonstration of any relationship among the high molecular weight bands with any one of the classical *Y. pestis* plasmid should await the use of specific molecular probes. Studies to disclose specific phenotypic traits associated with strains harbouring the additional cryptic bands are in progress in our laboratory.

ACKNOWLEGMENTS

We acknowledge the skilful technical assistance of Mrs Yara Nagasawa. Rosangela Célia Leandro de Farias and Marise Sobreira Bezerra da Silva were recipients of Scholarships from the Fundação de Amparo à Pesquisa do Estado de Pernambuco (FACEPE). Tereza Cristina Arcanjo Leal was recipient of a Fellowship from the Brazilian Research Council (CNPq).

RESUMO

Perfil plasmidial de cepas de *Yersinia pestis* isoladas no Nordeste do Brasil

Foi analisada a composição plasmidial de 182 cepas de *Yersinia pestis* isoladas em seis focos naturais de peste do Nordeste do Brasil. Cento e seis cepas (61,5 %) apresentaram o perfil plasmidial clássico composto de três plasmídeos bem caracterizados. Cinquenta e seis cepas (33,3 %) perderam pelo menos um deles e 20 cepas (11,0 %) apresentaram plasmídeos crípticos adicionais. Estas variações no perfil plasmidial foram observadas entre cepas originadas dos focos da Chapada do Araripe, Planalto da Borborema, Serra da Ibiapaba e Bahia enquanto todas as cepas analisadas provenientes dos focos de Triunfo e Serra de Baturité apresentaram o perfil plasmidial clássico.

Palavras-chave: *Y. pestis*, plasmídios, peste.

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Revista de Microbiologia (1997) 28:46-48
ISSN 0001-3714

CHARACTERIZATION OF *BACILLUS CEREUS* ISOLATED FROM CORN AND CASSAVA FLOUR SAMPLES IN CURITIBA

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SHORT COMMUNICATION

ABSTRACT

Thirty one strains of *Bacillus cereus*, detected in corn and cassava flour obtained at the local market in Curitiba, PR, were characterized by their biochemical and toxicological properties. All strains were positive for tests of hemolysin, phospholipase C, and mouse lethal toxin. The average lethal doses (LD50) ranged from 3.0×10^6 to 6.0×10^6 CFU/ml. All strains grew in nutrient broth supplemented with glucose, starch, or casein. None of them assimilated lactose or mannitol. All the 31 cultures of *Bacillus cereus* were inhibited when 2% citrate was used as a sole carbon source. Since *Bacillus cereus* is a facultative anaerobe, citrate does not prevent growth when the bacteria is cultivated in the presence of other carbon sources such as starch, glucose or casein. Citrate inhibition of F1-ATPase activity is discussed.

Key words: *Bacillus cereus*, toxins, citrate.

In a previous work we evaluated the microbial contamination of corn and cassava flours, obtained at local market in Curitiba, PR, and the presence of *Bacillus cereus* was observed (4). Such a presence in these dehydrated foods indicates, mainly, contamination by environmental sources (8).

Bacillus cereus was analyzed because it is considered an ubiquitous organism that causes diarrheal and emetic food poisoning syndromes, as well as a variety of infections (2). It has been associated with non-gastrointestinal infections including mastitis in cattle (7) and ophthalmia (9).

Bacillus cereus may cause food poisoning when the food contains a large number of viable cells, namely above 10^6 CFU/g (1). Food poisoning by *Bacillus cereus* is normally associated with toxin production (mouse lethal toxin, ß-hemolysin, and phospholipase C) (6).

We now characterize *Bacillus cereus* using biochemical and toxicological tests. The effect of some carbon sources on the growth of different strains of *Bacillus cereus* in culture media was evaluated. The effect of citrate as inhibitor of bacterial growth was also studied.

Isolation of *Bacillus cereus* was performed according to Speck (12) and Sneath (11), on 95 food samples, comprising 60 samples of corn flour (12 batches with 5 different packets of each) and 35 samples of cassava flour (7 batches with 5 different packets of each), obtained in stores in Curitiba, PR, in the period of August, 1993 to July, 1994.

Hemolytic activity was determined using sheep blood agar plates, indicated by a clear zone of complete hemolysis surrounding the colonies, after incubation at 30°C for 24 hr. Phospholipase C activity was detected in nutrient broth with egg yolk emulsion,

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which rendered the broth thick and milky, after incubation at 30°C for 24 hr. For the tests of mouse lethal toxin, one ml of a BHIG culture (Brain Heart Infusion broth, supplemented with glucose) obtained after incubation at 30°C for 17 hr, was injected into the intraperitoneal cavities of 5 mice of approximately 30 g (6). A high live cell concentration was used (10^8 - 10^9 CFU/ml). Average lethal doses (LD50) were estimated using the Reed-Muench method (10).

All strains were assayed for acid production from carbohydrates in a basal medium (Difco). The carbohydrates (0.5%, w/v) were mannitol, lactose and glucose. For assays of casein and starch utilization, all strains were grown in a agar-medium with 0.5% (w/v) of the carbon source (3).

All strains were cultivated at 28°C in a medium containing either 0.1% glucose (control), 1% citrate or 2% citrate as a carbon source, using a New Brunswick rotatory shaker at 150 rpm (3). Samples were collected asseptically after 0, 2, 4, 6, 8, and 10 hr of incubation, and the bacterial growth monitored spectrophotometrically at 550 nm.

All strains were positive for tests of hemolysin, phospholipase C, and mouse lethal toxin. The death of mice occurred within 30 min to 6 hr after injection. The median mouse lethal doses (LD50) of the strains ranged from 3.0×10^6 to 6.0×10^6 CFU/ml.

Regarding the utilization of different carbon source for growth in the culture media, none of the strains was positive for lactose and mannitol assimilation. All of them gave positive results for glucose (under aerobic and anaerobic conditions), starch and casein.

The effect of citrate (1 and 2%) on bacterial growth was compared to the effect of glucose 0.1%, used as a control. The results are illustrated in Fig. 1. All strains of *Bacillus cereus* were inhibited by 2% citrate. Higuti *et al.* showed that *Bacillus cereus*, strain ATCC 145798, could not attain normal growth in the presence of citrate above 40 mM, probably due to the inhibition of ATPase activity. It was observed that citrate lowers the affinity between ATPase and ATP, and high concentration of ATP does not counteract the inhibition (5). Since *Bacillus cereus* is a facultative anaerobe, citrate does not prevent growth when the bacteria is cultivated in the presence of other carbon source such as starch, glucose or casein. The bacteria is able to survive because it uses ATP produced in the glycolytic pathway.

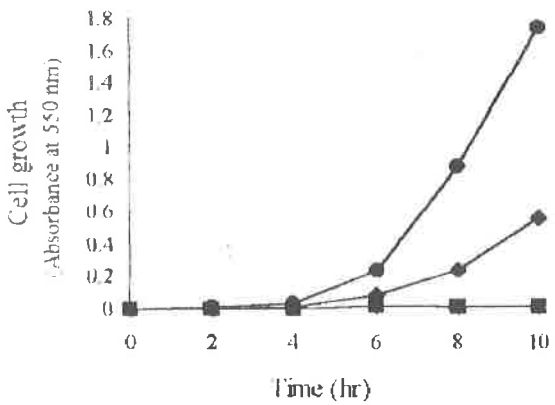

Figure 1. *Bacillus cereus* growth in the presence of glucose and citrate, as sole carbon sources.
● - 0.1% glucose, ◆ - 1% citrate, ■ - 2% citrate

ACKNOWLEGMENTS

AJN wishes to thank M. Ohi and A.C. Tanaka for their help in the LD50 determinations.

RESUMO

Caracterização de *Bacillus cereus* isolado de amostras de fubá de milho e farinha de mandioca em Curitiba

Cepas de *Bacillus cereus* isoladas de fubá de milho e farinha de mandioca, adquiridos no mercado varejista da cidade de Curitiba, PR, foram caracterizadas por suas propriedades bioquímicas e toxicológicas. Todas as cepas foram positivas para os testes de hemolisina, fosfolipase C e toxina letal para camundongos. A dose letal média para toxina letal para camundongos (DL50) variou entre 3.0×10^6 a 6.0×10^6 UFC/ml. Todas a cepas cresceram no meio básico de cultura, suplementado com glucose, amido ou caseína, porém, foram negativas para a assimilação de lactose ou manitol. Todas as cepas foram incapazes de crescer em citrato 2%, como única fonte de carbono. Como o *Bacillus cereus* é um anaeróbio facultativo, o citrato não impede sua proliferação quando a bactéria é cultivada na presença de outras fontes de carbono como glicose, amido ou caseína. A inibição da F1-ATPase pelo citrato é discutida.

Palavras-chave: *Bacillus cereus,* toxinas, citrato.

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Revista de Microbiologia (1997) 28:49-54
ISSN 0001-3714

VIRULENCE FACTORS IN MOTILE *AEROMONAS* SPP ISOLATED FROM VEGETABLES

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ABSTRACT

The pathogenicity of 48 strains of *A. caviae* and two of *A. hydrophila* isolated from vegetables (escarole, water-cress, lettuce) was evaluated by adhesion and invasion tests as well as the production of enterotoxins *in vivo* (suckling mouse test) and *in vitro* (HEp-2 cells assay). *A. hydrophila* did not adhere or invade HEp-2 cells, probably due to the destruction of the cell monolayer by the cytotoxin that was detected in the *in vitro* test. Out of the *A. caviae* strains tested, 62.5% were classified as adhesive and only 2% from the total of strains were able to invade cells. In relation to enterotoxin production, 34% were positive *in vivo* and only 10% *in vitro*. All strains were negative in the suicidal phenomenon test which did not show any correlation with the pathogenicity of isolated strains. Based on the results obtained in this study we concluded that *Aeromonas* spp isolated from vegetables may represent risk to consumers' health.

Key Words: *Aeromonas* spp, virulence factors, pathogens, vegetables

INTRODUCTION

Aeromonas spp are Gram-negative, facultative anaerobes that belong to the *Vibrionaceae* family. They are able to grow at low temperatures (4°C) (35), commonly used for storage of fresh and raw foods. These bacteria are widespread in nature and are most commonly associated with water (3). So it is not surprising that they have also been isolated from fish and crustaceans (15, 20, 23, 24, 28, 39) and vegetables (6, 37).

Aeromonas spp may be significant in food hygiene as potential agents of foodborne illness since they were isolated from retailed fresh food of vegetable origin and these microorganisms are able to grow competitively in foods at refrigeration temperatures.

The organism has been recognized as a pathogen associated with several categories of human diseases, including gastrointestinal and extraintestinal infections. The gastroenteritis can range from watery (21) to bloody diarrhea (12) and besides, there are reports of chronic colitis of prolonged duration (11). The extraintestinal infections include water or soil contaminated wound infections with rapid onset of cellulitis, septicemia in immunocompromised hosts, urinary tract infection, myositis, conjunctivitis, osteomyelitis, meningitis, endocarditis, peritonitis and aspiration pneumonia (18).

Aeromonas spp strains are able to produce a variety of biologically active extracellular substances, including haemolysins, enterotoxins, adhesins and enzymes. Some strains are also adhesive and invasive and may produce haemagglutinins possibly associated with diarrhea. Namdari and Bottone (29) suggested the possible existence of an association between pathogenicity and *Aeromonas* spp suicidal capacity. However, there is not a general consensus about the role of these factors in enteropathogenicity (33).

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The increasing number of reports in recent years implicating *Aeromonas* spp as the etiologic agents of acute diarrhea suggests that these organisms are more important in relation to what is currently being appreciated (23). However, relatively little is known about the occurrence of these organisms in food and their significance as foodborne pathogens.

The aim of this study was to ascertain the potential virulence of *Aeromonas* strains isolated from vegetable samples through their adhesive and invasive abilities and *in vivo* and *in vitro* production of thermo-labile enterotoxins.

MATERIALS AND METHODS

Bacterial strains. Fifty *Aeromonas* spp strains (2 *A. hydrophila* and 48 *A. caviae*), isolated from vegetable samples (lettuce, water-cress and escarole), (37) were tested. The two *A. hydrophila* strains were from water-cress and lettuce. Among *A. caviae*, 11 strains were from lettuce, 10 from escarole and 27 from water-cress.

Virulence factors. All strains were submitted to the following tests:

Adhesion assay. Strains were grown on blood agar (BA) (Difco) plates incubated at 28°C for 18-24 h. In order to prepare overnight cultures, single colonies were cultured in 10 ml of brain heart infusion broth (BHIB) (Difco) and grown statically for 16-18h at 28°C. Log-phase cultures were prepared by adding 0.5 ml of the overnight culture to 10 ml BHIB followed by static incubation for 3-5 h at 28°C. These cultures were diluted in Hanks balanced salt solution (HBSS) to a final concentration of 2-3 x 10^6 CFU/ml as previously determined by serial dilution and plating on tryptic soy agar (TSA) (Difco) incubated at 28°C for 24 h. One ml of the bacterial cultures was added to the wells of a 24-well tray, containing glass coverslips recovered with a semiconfluent monolayer of HEp-2 cells grown for 20 h in Minimal Eagle Medium (MEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (Fazenda Pig) and washed three times with 2 ml HBSS. Three wells were used for each strain. After incubation for 90 min. at 37°C in a 5% CO_2 atmosphere, non-adherent bacteria were removed from the infected monolayers by washing four times with 2 ml HBSS. The coverslips were removed from the tray, fixed in methanol:acetic acid solution(3:1) for 5 min.; stained by Gram's method and then mounted on glass microscope slides. Adhesion was assessed by bright-field microscope at magnification of 1000 x under oil immersion (7). The percentage of infected cells was determined for every monolayer and the number of bacteria associated to twenty-five randomly chosen infected cells was counted. Thus, a total of 75 counts of infected cells was obtained for each strain. The average count of the number of bacteria per infected cell was calculated. The resultant average was multiplied by the average number of infected cells in the three coverslips, yielding a score of bacteria per HEp-2 cell. Strains presenting scores from 1 to 10 bacteria/cell were classified as "low adherers"; from 10 to 20 bacteria/cell strains as "adherers" and more than 20 bacteria/cell as "high adherers" (7). An adherent *Escherichia coli* strain was used as positive control.

Invasion assay. Strains were grown on BA plates incubated at 28°C overnight. Single colonies were inoculated into 10 ml BHI (Difco) and grown statically for 16-18 h at 28°C. Bacteria were suspended in MEM with 10% FCS to a final concentration of approximately 5 x 10^5 cells/ml. This concentration was checked by plating decimal dilutions on TSA (Difco). The plates were incubated at 28°C for 24 h. One ml of the bacterial cultures was added to the wells of a 24-well tray (Costar Corporation - Cambridge) containing a semiconfluent monolayer of 5.0 x 10^4 to 1.0 x 10^5 HEp-2 cells/ml which were grown for 18 h in a humidified 5% CO_2 incubator. The trays were incubated for 3 h at 37°C in 5% CO_2 atmosphere, and then washed *in situ* three times with 2 ml HBSS. Fresh MEM containing 10 μg/ml gentamicin was added to the monolayers which were incubated for additional 2 h, under the same conditions, and again washed for 3 times with HBSS, as recommended by Watson *et al.*(1985). One ml of MEM containing 5% FCS but without gentamicin was added to each well. The monolayers were incubated for a further 2-hour period at 37°C with 5% CO_2 and washed three times with HBSS. The integrity of the monolayer was then checked, and 1 ml of lysing solution containing 0.01 M NaH_2PO_4 (Baker), 1% Tween 20 (Merck) and 0.025% trypsin (Merck) (wt/vol) at pH 8.0 was added to each well and incubated for 30 minutes under the same conditions (37). The lysate and the decimal dilutions made in BHI broth were plated onto BHI agar (Difco) to determine the number of CFU per milliliter. The strain was classified as non-invasive if bacteria were not recovered or were recovered only from the undiluted lysate, which means that the growth did not exceed 2 x 10^3 CFU/ml. Strains with growth of > 5 x 10^6 CFU/ml were classified as invasive. When the number of recovered bacteria ranged from 2 x 10^3 to 5

x 10^5 CFU/ml, the strain was retested. An invasive *E. coli* strain was used as positive control.

***In vivo* detection of thermo-labile enterotoxin.** The enterotoxin was detected *in vivo* by the suckling-mouse test, using isogenic mouse, strain C_3He/Pas. Strains were inoculated in tubes containing BHIB and incubated at 28°C for 18h. Cultures were centrifuged (Celm centrifuge - Mod. LS-II) at 3,000 rpm for 30 min. and the supernatants were filtered through 0.45 µm membrane filters (Millipore) (4), and the resulting cell-free preparations were kept at -70°C. Test supernatants (100 µl) containing 0.05% Pontamine Sky Blue (Merck) were inoculated intragastrically into 2- to 4-day old mice using a 1-ml seringe. Three mice were used in each test. After 4 h at room temperature, the animals were killed with chloroform (Synth) and their bowels were removed and weighed. The ratio between the intestinal weight (IW) and the remaining body weight (BW) was calculated. The test was considered positive when the ratio was higher than 0.085. In each assay, an *Aeromonas hydrophila* strain was used as positive control. Sterile BHIB containing 0.05% Pontamine Sky Blue was the negative control (1, 25, 26).

***In vitro* detection of thermo-labile enterotoxin.** The same supernatants obtained for the suckling-mouse test were used for the visualization of the cytotoxic effect of the enterotoxins. These supernatants were diluted 1:5, 1:8 and 1:10 in BHIB. 0.1 ml of each dilution was inoculated into the wells of a 96-wells tray (Nunc - Inter Med- Denmark) containing a monolayer of HEp-2 cells which were grown for 24 h in a humidified 5% CO_2 incubator at 37°C. Each strain was tested in duplicate. The tray was incubated for 24 h at 37°C in a humidified 5% CO_2 incubator. After this period, the cells were examined in bright-light microscope in order to verify the cytotoxic effect (6, 17, 34). An *A. hydrophila* strain was used as positive control and sterile BHIB and its dilutions as negative controls.

Suicidal phenomenon. The suicidal tendency of *Aeromonas* isolates was assessed as described by Nandari and Bottone (31) with some modifications. Briefly, all isolates were plated onto Columbia agar (Difco) containing 5% defibrinated sheep blood and incubated at 28°C for 24h. Each strain was transferred to two tubes (controls) containing Nutrient Broth (NB)(Difco) and to two tubes containing NB plus 0.5% glucose (Merck). One tube of each set was incubated at 37°C for 24h and the remaining ones at 30°C for 24h. The test was considered positive when the control tube was visually muddy and the test tube was clear, with sedimented cells .

RESULTS AND DISCUSSION

Adhesion assay. Among the fifty *Aeromonas* strains that were submitted to the adhesion assay, 30 (60%) adhered to HEp-2 cells. All the adherent strains were identified as *A.caviae*. The two *A. hydrophila* strains were negative. Considering only the 48 *A.caviae* strains, 62.5% of them were positive.

Other authors also observed that *A. caviae* is capable of adhering to HEp-2 cells, though in a lower percentage (7, 14, 19). Carrelo *et al.* (7) submitted eight *A. caviae* strains to adhesion assay and observed that 37.5% presented positivity. Grey and Kirov (14) tested 60 *Aeromonas* strains of clinical, water and food origin and noted a similar positivity of adhesion (40 and 41%, respectively) in clinical and water strains. On the other hand, these authors observed a lower positivity for adhesion in strains isolated from food (18%). They also described different patterns of adhesion among different species. *A. veronii* biotype *sobria* showed the highest proportion (58%) of adhesive strains. At the same time, 33% of *A. caviae*, 11% of *A. hydrophila* and none of the non-classified strains were adherent. Kirov *et al.* (19) did not detect pili in clinical isolates. On the other hand, a great amount of this structure was detected in environmental strains, what can explain the low percentage of positivity detected in clinical samples and the high positivity showed by strains isolated from vegetables.

The positivity of adhesion of *A. caviae* to HEp-2 cells in relation to the number of bacteria/cell is shown in Table 1. The majority of isolates were classified as adherers or high adherers, with more than 10 bacterial/HEp-2 cell. The maximum adhesion level was 47 bacteria/cell.

Table 1 also summarizes *Aeromonas caviae* adhesion in relation to the source of the isolate. From 10 *Aeromonas* strains isolated from escarole, seven adhered to HEp-2 cells to some degree, and the majority (57%) of the isolates were high adherers (20 bacteria/cell). Among strains isolated from watercress, 17 (62,9 %) presented adherence to some degree. Among these, 14 (82.4%) presented more than 10 bacteria/cell. *A. caviae* strains isolated from lettuce showed the lowest adherence pattern: 54.6% of strains were positive and among them 50% were classified as low adherers. These results disagree with the ones of Carrelo *et al.* (7) and Namdari and Bottone (30) who

Table 1 - Adhesion of *Aeromonas caviae* strains to HEp-2 cells according to the source of the isolate

Adhesion (bacteria/ cell)	Source						total	
	lettuce		water-cress		escarole			
	n	%	n	%	n	%	n	%
< 1	5	45.4	10	37.1	3	30.0	18	37.5
1 - 10	3	27.3	3	11.1	0	0.0	6	12.5
11-20	1	9.1	7	25.9	3	30.0	11	22.9
20	2	18.2	7	25.9	4	40.0	13	27.1
Total	11	100.0	27	100.0	10	100.0	48	100.0

Table 2 - Positivity for *in vivo* and *in vitro* enterotoxin production by *A.hydrophila* and *A. caviae* strains

species	positivity for enterotoxins production	
	in vivo n (%)	*in vivo* n (%)
A. hydrophila	2 (100)	2 (100)
A. caviae	16 (34)	5 (10)
TOTAL	18 (38)	7 (14)

found 7% of strains with low level of adhesion .On the other hand, our results are in accordance with the observations of Carrelo *et al.* (7) and Gery and Kirov (14), who concluded that *Aeromonas* strains are able to adhere to HEp-2 cells in tissue culture, and that the adhesion assay is relatively rapid, easy to perform, and highly reproducible.

A. caviae is not considered an enteropathogen due to the lack of virulence factors such as production of enterotoxins, cytotoxins, or invasiveness ability (16, 19, 38). However, Namdari and Bottone (30) suggested that while absolute relevance can not be ascribed to *in vitro* HEp-2 cells adherence, a correlation between enteropathogenicity and *in vitro* adherence does exist for numerous other bacterial species. So the authors proposed that *A. caviae* may be an enteric pathogen as well as *A. hydrophila* and *A. sobria*. This proposal was also made in 1985 by Altwegg (2), who suggested that the detection of *A. caviae* in some geographic areas is related to diarrhea. Besides, this species was recovered as the sole potential enteric pathogen from stools of 14 children with watery diarrhea by Namdari and Bottone (30). All the *A. caviae* isolates adhered to HEp-2 cells without internalization. Thus, these authors concluded that the adherence could act as a virulence mechanism for *A. caviae* in the same way as it happens with *E. coli*.

From this point of view, we believe that *A. caviae* strains isolated from vegetables could be considered as pathogens since they showed a great percentage of positivity in the adhesion assay.

Invasion assay. From 48 isolates identified as *A. caviae*, one had the ability to adhere and invade the HEp-2 cells monolayer. Many authors have reported the lack of invasive ability of *A. cavie*. Janda *et al.* (16) considered this species as the less invasive as it was rarely associated with bacteremia in their patients. Watson *et al.* (38) worked with 69 fecal isolates of *Aeromonas* spp and correlated the invasiveness with biotype. The authors observed that of the 18 invasive strains, 16 were *A. sobria* and two were *A. hydrophila*. No invasive strains were found among the *A. caviae* strains. Gray *et al.* (13), who studied the incidence of virulence factors in mesophilic *Aeromonas* species isolated from animals and their environment, found that 36.4% of *A. sobria*, 21.4% of *A. hydrophila* and 13.6% of *A. caviae* strains were invasive.

Although the two *A.hydrophila* strains tested were classified as non invasive, both produced toxin with cytotoxic effects. In the invasion assay performed in this work, *Aeromonas* spp remained for 7 h in contact with HEp-2 cells. This time could be enough for the production of toxins, since Namdari and Bottone (30) detected *A.hydrophila* and *A.sobria* toxin activity in the supernatant fluids as early as 8 h postinoculation. This cytotoxin could possibly alter the cellular membrane, allowing the gentamicin penetration, which eliminates intra and extracellular bacteria.

Besides, all the strains tested in this study were isolated from vegetable samples and the invasiveness ability seems to be unusual among environmental isolates and common in isolates from clinical samples (5, 22).

***In vivo* and *in vitro* detection of enterotoxin.** Table 2 shows the percentage of *Aeromonas* strains that produced enterotoxins *in vivo* (suckling-mouse test) and *in vitro* (cytotoxic effect). The two strains of *A.hydrophila* (100%) produced thermo-labile toxins that acted *in vivo* and *in vitro*. Thirty-four percent of *A. caviae* isolates produced thermo-labile toxins detected *in vivo* tests and 10% *in vitro* tests. These results are similar to those reported by other authors who studied clinical strains (9, 13, 17, 18) or isolates from foods (10, 34).

Namdari and Bottone (30), in 1990, studied 21 stools and environmental *A. caviae* isolates for cytopathogenicity in cultured HEp-2 cells and for enteropathogenicity by suckling-mouse test. They observed that toxin was produced by *A. caviae* in glucose-free double strength TSB but not in double

strength TSB containing 6.4% glucose. The authors suggested that glucose apparently repressed *A. caviae* cytotoxin production but not that of *A. hydrophila* or *A. sobria*. That is why single-strength TSB and BHI were adequate to demonstrate toxin production by *A. hydrophila* and *A. sobria* but not by *A. caviae*. They postulated that the presence of glucose may suppress toxin production via an identical mechanism or as a consequence of slowing cell growth and toxin production below a detectable threshold.

In the present study, 16 (34%) *A. caviae* strains grown in BHI broth produced enterotoxin as detected by the suckling-mouse test. We believe that our *A. caviae* strains were not inhibited by the glucose present in this culture medium since none of them were suicidal in the suicide phenomenon test.

The positivity for enterotoxin production (34%) was higher than the results found by other authors. Watson *et al.* (1985) (38) analyzed 68 strains of different sources and found that 11% were positive in the suckling mouse test. Figura *et al.* (1986) (9) tested only one *A.caviae* strain, which was negative in this test. Majeed *et al.* (1989b) (28) observed that 8% of *A.caviae* strains isolated from different sources (water, feces, chickens) were able to cause fluid accumulation in this test. The higher percentage of toxin producing strains found in our work can be explained by the different mouse lineage used. In a previous work performed in our laboratory, 16 different isogenic mouse lineages were inoculated with *Aeromonas* enterotoxin and the C_3He/Pas lineage gave the best results. It seems that different mouse lineages can produce different responses in the suckling mouse test.

Suicide phenomenon. Some authors (26, 32) described a deleterious effect of glucose in the growth of *A hydrophila* group *caviae* and a variable response to the presence of this sugar in the *A hydrophila* group *hydrophila* and *sobria* strains. This variation was not detected in the present work. This agrees with Rodrigues (36), who analyzed different strains of *Aeromonas* isolated from food, and with Mores (27), who worked with *Aeromonas* isolated from fish samples.

Although Namdari and Bottone (29) proposed an association between suicide phenomenon and lethality of rats which was responsible for the classification of the suicide phenomenon as a possible virulence factor of *Aeromonas* spp, the results of the present work do not suggest this association for strains isolated from food samples.

Namdari and Bottone (31) suggested that acetic acid-mediated phenomenon in mesophilic aeromonas in conjunction with tests for aerogenicity and esculin hydrolysis could serve as the basis for species identification. However, our results do not support these authors data, since all the strains that were tested were not suicidal.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. L.R. Trabulsi, Department of Microbiology, São Paulo University, Brazil, and to Dr. R. Toledo, Department of Microbiology, Federal University of São Paulo, Brazil, for supplying the strains of *E. coli* and *Aeromonas* sp, respectively. We also thank Dr. C. Harsi, Department of Microbiology, São Paulo University, Brazil, for providing HEp-2 cells, T. Marques, Department of Immunology, São Paulo University, Brazil, for supplying the isogenic mice and C.A. Silva, Department of Microbiology, São Paulo University, Brazil, for technical help with the animals used in this study. This work received financial support from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo).

RESUMO

Fatores de virulência em *Aeromonas* spp. móveis isoladas de hortaliças

A patogenicidade de 48 cepas de *A. caviae* e de duas de *A. hydrophila* isoladas de vegetais (alface, agrião e escarola) foi verificada através de testes de adesão, de invasão e de produção de enterotoxinas *in vivo* (teste de Dean) e *in vitro* (cultura de células HEp-2). As duas cepas de *A. hydrophila* apresentaram resposta negativa nos testes de invasão e de adesão. Também foram detectadas *in vivo* enterotoxinas produzidas por estas cepas . Das cepas de *A. caviae,* 62,5% foram capazes de aderir à célula HEp-2, sendo que 2% do total de cepas invadiram esta linhagem celular. Com relação à produção de enterotoxina por *A. caviae*, 34% foram positivas *in vivo* e somente 10% *in vitro*. Todas as cepas foram negativas no teste do fenômeno suicida, que não se relacionou com a patogenicidade das cepas isoladas. Assim, de modo geral, as *Aeromonas* isoladas de hortaliças apresentaram algum fator de virulência, podendo vir a representar risco à saúde de seus consumidores.

Palavras-chave: *Aeromonas* spp, fatores de virulência, patógeno, hortaliças

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Revista de Microbiologia (1997) 28:55-60
ISSN 0001-3714

VIRULENCE RELATED CHARACTERISTICS OF *ESCHERICHIA COLI* FROM SOWS WITH MASTITIS-METRITIS-AGALACTIA (MMA) SYNDROME

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ABSTRACT

Virulence related biological characteristics of thirty *Escherichia coli* strains isolated from sows with Mastitis-Metritis-Agalactia (MMA) syndrome were examined. Eighteen (60%) *E.coli* strains agglutinated with guinea pig RBC in the absence of D-mannose. Eleven strains possessed type 1 fimbria, as shown by seroagglutination tests. None of the *E.coli* strains expressed P1 and F165 fimbrial antigens. None of them produced enterotoxins (STa, STb and LT1), cytotoxins (VTs and CNFs) or were hemolytic (α and enterohemolysin). Seventeen *E.coli* strains produced aerobactin and fourteen produced colicin V. Twenty-five isolates were serum resistant. None of the isolates adhered to pig kidney cells (SK6, PK15 and C17) or to HeLa cells. These results suggest that the pathogenicity of these *E.coli* strains is multifactorial and further molecular studies are required to determine markers of virulence in MMA-*E.coli*.

Key-words: *Escherichia coli*, virulence factors, Mastitis-Metritis-Agalactia (MMA) syndrome, sows.

INTRODUCTION

The Mastitis-Metritis-Agalactia (MMA) syndrome, a non-epidemic disease in sows, is a puerperal infection causing endometritis and inflammation in mammary glands. This inflammation causes agalactia of the sows, which leads to the incapability of the piglets to feed, causing the deprivation of the immunoglobulins provided by colostrum (10). This lack of nourishment is responsible for a mortality rate of about 80% among young piglets which cannot be protected against possible environmental infectious bacteria. Clinically, the sows present increased respiratory rate, high rectal temperature and mastitis. Visual evidence of mammary involvement is frequently observed as a slight enlargement of one or more mammary glands, anorexia and central nervous system depression. The etiology of lactation failure is still unknown, although some suggestions can be found in the available literature (3,21,25).

Avila *et al.* detected *E.coli*, followed by *Arizona sp*, *Staphylococcus sp* and *Salmonella sp.*, in approximately 70% of genital cultures of 27 sows with clinical signs of mastitis (2). Thomas and MacLean attributed the high incidence of metritis caused by *E.coli* in sows to an increase of these bacteria in the genital tract, since the resistance of these animals is decreased by parturition (39). Other authors observed similar results (32, 33). *E.coli* strains isolated from milk of sows with MMA presented K88, K99, 987P and other specific fimbrial antigens on the bacterial surface. Nevertheless, the role of *E.coli* in MMM syndrome is not clear yet.

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The purpose of this work was to study the biological characteristics of *E.coli* strains isolated from sows with clinical signs of MMA in Brazil.

MATERIALS AND METHODS

Bacterial strains: A total of 30 *E.coli* strains, isolated in the region of Campinas, SP, Brazil, from mammal secretions of sows with clinical signs of MMA, were used in this study. The strains were stored at -20°C in Brain Heart Infusion (BHI - DIFCO) with 15% glycerol.

Antisera: The rabbit antisera produced against the adhesins studied in this work were prepared as recommended by Edwards and Ewing (15) for preparation of O:K antisera. Specific fimbrial antigens antisera were prepared by exhaustive absorption with homologue *E.coli* strains grown at 16°C.

Colicin assay: For the detection of colicins, the MMA *E.coli* strains were cultured in BHI (DIFCO) and assayed as described by Fantinatti *et al* (18).

Microhemagglutination (MH) test: The MMA *E.coli* strains were grown on media recommended for production of adhesins (13, 41) and suspended in phosphate buffered saline (PBS) to 10^8 cells/ml. MH test was carried out in the presence of D- mannose (mannose resistant microhemagglutination - MRMH) and abscence of D-mannose (mannose sensitive microhemagglutination - MSMH), as described by Jones and Rutter (22). The MMA *E.coli* strains were grown for 24hs at 37°C in the culture media recommended for each adhesin and the tests were performed with specific erythrocytes as described previously (13).

Hemolysins production: The MMA *E.coli* strains were plated on blood agar base plus 5% (v/v) defribrinated sheep erythrocytes and incubated at 37°C overnight. Hemolysin production was detected by the presence of a clear halo around the colonies. For enterohemolysin detection, MMA *E.coli* strains were plated on blood agar base containing $CaCl_2$ and 5% washed defribinated sheep erythrocytes and incubated overnight at 37°C (4).

Enterotoxins assays: For the detection of heat-stable enterotoxin (STa) and heat-labile enterotoxin (LT1), MMA *E.coli* strains were grown in CAYE at 37°C for 18 hours with shaking (16). The culture supernatants were tested by the radial immunehemolysis test for LT1 detection (43) and the suckling mice test for STa detection (12). The strains were also submitted to DNA probe tests for production of enterotoxins STb and LT1, as described by Blanco *et al* (6).

Cytotoxins assay: For detection of verocytotoxin (VT), MMA *E.coli* strains were grown in Trypticase Soy Broth (TSB - DIFCO) at 37°C for 18hours, with shaking (150rpm). For production of cytotoxic necrotizinig factor (CNF), *E.coli* strains were cultured in the same conditions described above, in the presence of mitomicin C (0.25μg/ml of medium) as described by Blanco *et al* (6).

Aerobactin production: For aerobactin detection, the method described by Carboneth and Williams (7) was used. Minimal medium M9 containing α,α dipyridil (160μM) to chelate available iron, and supplemented with succinate 0.5% (w/v), thiamine (50μl/ml) and casaminoacids 0.5% (w/v), incubated for 24 hours at 37°C with aeration was used. *E.coli* strains K12 LG 1315 and K12 C600 were used as positive and negative controls, respectively.

Congo Red Binding Test: The Congo Red binding capacity of the MMA *E.coli* strains was examined in Trypticase Soy Agar (TSA -OXOID) containing 0.6% yeast extract and 0.003% Congo Red (DIFCO), as described by Qadri *et al.* (43).

Bacterial Motility test: The motility of MMA *E.coli* strains was tested in semi-solid Cystine Tryptic Agar (CTA) and was observed for one week at 37°C.

Seroagglutination test: The expression of known fimbriae was assayed by the seroagglutination test on glass slide with specific K88, 987P, K99, F41, F42, F18ab (F107), F18ac (2134P), F165, P1 and type 1 fimbria antisera.

***In vitro* bacterial adherence assays:** *In vitro* adherence assays of *E.coli* strains were carried out as described by Scalestsky *et al.* (38), using HeLa and SK6, PK15 and C17 swine kidney cells, grown in Leightons tubes in Eagle minimal medium (MEM) containing 10% bovine fetal serum (BFS).

Serum resistance assay: Assays to determine resistance of MMA *E.coli* strains to normal swine serum were carried out following the quantitative method described by Fantinatti *et al* (18) using Peptone Glucose (PG) medium (29). The rate of growth and hence the action of the serum on the bacteria were measured using the time 0 hour without serum as the control. *E.coli* LG 1315 and *E.coli* 1522 were used as positive and negative controls, respectively.

RESULTS

The results of hemagglutination tests are shown in Table 1. Eighteen (60.0%) MMA *E.coli* strains

Table 1. Microhemagglutination (MH) of MMA *E.coli* strains using different kinds of erythrocytes

erythrocytes	positive strains/ total (%)
guinea pig (MSMH*)	18/30 (60.0)
guinea pig (MRMH**)	4/30 (13.3)
chicken	2/30 (6.7)
equine	2/30 (6.7)
bovine	2/30 (6.7)
human	0/30

MSMH*: D-mannose sensitive microhemagglutination
MRMH**: D-mannose resistant microhemagglutination

Table 2 - Seroagglutination of MMA *E.coli* strains for detection of adhesins

antisera	positive strains/ total (%)
a-K88	4/30 (13.3)
a-987P	4/30 (13.3)
a-K99	5/30 (16.7)
a-F41	8/30 (26.7)
a-F42	5/30 (16.7)
a-F107 (F18ab)	2/30 (6.7)
a-2134P (F18ac)	6/30 (20.0)
a-P1	0/30 (0)
a-F165	0/30 (0)
a-type 1 fimbria	11/30 (36.7)

agglutinated with guinea pig RBC in the absence of D-mannose (MSMH). Among them, only 4 strains (13.3%) hemagglutinated with guinea pig erythrocytes in the presence of D-mannose (MRMH). Two strains agglutinated with chicken, equine and bovine RBC in the presence of D-mannose (MRMH). None of the *E.coli* strains agglutinated with human erythrocytes (MRMH).

As shown in Table 2, the MMA *E.coli* strains did not express the P1 and F165 fimbrial antigens. The frequency of fimbrial antigens related to enterotoxigenic *E.coli* (ETEC) (K88, 987P, K99, F41 and F42) ranged from 4 (13.3%) to 8 (26.7%) strains. The two new fimbrial antigens related to *E.coli* strains isolated from enterotoxigenic edema disease (ED) in pigs and also to ETEC strains, called F107 (F18ab) and 2134P (F18ac), were detected in two strains. Eleven MMA *E.coli* strains (36.7%) possessed type 1 fimbria.

Escherichia coli strains isolated from sows with Mastitis-Metritis-Agalactia neither produced enterotoxins (STa, STb and LT1) nor cytotoxins (VTs and CNFs). Twenty-one (70%) of the MMA *E.coli* strains produced colicin and fourteen (66.7%) of them were classified as colicin V. Seventeen (56.7%) strains produced aerobactin (Table 3).

Table 3. Virulence characteristics of MMA *E.coli* strains

virulence factor	positive strains/ total (%)
colicins	21/30 (70.0)
colicin V	14/21 (66.7)
Aerobactin	17/30 (56.7)
VT*	0/30 (0)
CNF**	0/30 (0)
STa***	0/30 (0)
STb****	0/30 (0)
LT1*****	0/30 (0)
Hemolysins	
α-hemolysin	0/30 (0)
enterohemolysisn	0/30 (0)
motility	14/30 (46.7)
Congo Red binding	9/30 (30.0)
Serum resistance (resistants)	25/30 (83.3)

* -Verocytotoxin
**-Cytotoxic Necrotizing Factor
***-Heat-stable enterotoxin type I
****-Heat-stable enterotoxin type II
*****-Heat-labile enterotoxin type I

The MMA *E.coli* strains did not produce α-hemolysin or enterohemolysin (E-Hly) as assayed with washed defribinated sheep blood agar.

Nine (30%) *E.coli* strains were positive in the Congo Red binding test and fourteen (46.7%) were motile.

The resistance of MMA *E.coli* strains in early exponential culture to the bactericidal activity of normal pigs serum (NPS) was also determined. 83.3% of all MMA strains were resistant to NPS (Table 3).

The *in vitro* adherence to MMA *E.coli* strains was tested on HeLa and porcine kidney cells (SK6, PK15 and C17) but none of them was able to adhere to these cells (data not shown).

DISCUSSION

One of the best characterized biological factors of *E.coli* strains isolated from animals with enteric diseases are the surface structural adhesins, responsible for the adherence to the mucosal receptors of the host (11).

An adhesive antigen called 2134P, serologically distint from those described for porcine ETEC, was described in enterotoxigenic *E.coli* strains (8).

Although the F107 and 8199 fimbriae were described initially as the main adhesive factors of enterotoxemic *E.coli* (causative agent of edema disease in pigs) (14, 21), these adhesins were also observed in ETEC strains (29, 37). The F18ab and F18ac designations were proposed for F107 and 2134 adhesins respectively. Our results indicated that some MMA *E.coli* expressed the ETEC specific fimbrial antigens, in frequencies varying from 13.3% to 26.7% (Table 2). These data suggest that these adhesins may be important for bacterial colonization, leading to the MMA syndrome.

The F165 fimbrial antigen is considered an adhesive factor of *E.coli* associated to extraintestinal diseases (17). However this antigen was not observed in the MMA *E.coli* strains (Table 2).

Recently, Parreira (30) detected only fimbrial antigen F41 in 14% of *E. coli* strains isolated from chickens presenting Swollen Head Syndrom (SHS). However, the role of this adhesin in SHS remains unknown.

P1 fimbrial adhesin is the main colonization factor of pielonephritogenic *E.coli* in humans (14). Recently, this fimbrial adhesin was detected with high frequency in avian coliseptic disease, but nothing is known about the involvement of this adhesive factor in avian disease (40). Negative results of the MRMH test carried out with human erythrocytes possessing p^k antigens (Table 1) confirm the result obtained for agglutination with specific antiserum to detect P1 adhesin (Table 2). These data suggest that the P1 adhesin is not involved in MMA syndrome.

Type 1 fimbria was detected by seroagglutination test in 11 (36.7%) of 18 MSMH positive strains (Table 1). Our findings suggest that the positive reaction in MSMH with guinea pig erythrocytes does not indicate the presence of Type 1 fimbria. We reported similar results in enterotoxigenic *E.coli* strains isolated from adult bovines with diarrhea (9). The bovine strains, despite being MSMH positive and having similar molecular weight (c.a. 17 kDa), were serologically different.

The relatively high frequency of aerobactin and colicin production (Table 3) suggests that these biological properties may be related to virulence factors in MMA *E.coli*.

Yancey *et al.* (42), demonstrated experimentally that a great inoculum of *V.cholerae* is required to cause diarrhea in humans. Arp and Jansen (1) also demonstrated that the motility of the bacterium is important in avian bacterial infections. Our findings showed that 46.7% of the MMA *E.coli* strains were motile (Table 3), suggesting a role for motility in the infection.

Payne and Finkelstein (31) and Petersen (33) observed that *Yersinia*, *Shigella*, *Pasteurella* and invasive *E.coli* presented binding to Congo Red, suggesting that this capability could be one of the several markers of invasive bacterial virulence. Our results showed that 30% of the MMA *E.coli* strains bound to Congo Red. Thus, further studies at a molecular level are obviously necessary.

Another bacterium characteristic that can contribute to pathogenicity, mainly in sepsis infection, is serum resistance. Serum resistance can be attributed to the presence of outer membrane protein *TraT*, responsible for the surface exclusion phenomenon, enabling the bacterium that possesses it to be resistant to the bactericidal activity of the serum (28). It was demonstrated that the presence of a plasmid with multiple drug resistance would contribute to serum resistance (35, 36). The ability of a bacterial strain to resist the lethal effects of serum was determined in early exponential cultures that withstood the bactericidal activity mediated primarily by antibody, complement and lysozyme. Our data show that 83.3% of the MMA strains were resistant to the bactericidal activity of adult normal pig serum (NPS) and only 16.7% of the strains were sensitive to the serum, suggesting that serum resistance of bacteria can be considered an important virulence factor in MMA syndrome of sows.

Our findings (Table 3) revealed that MMA *E.coli* strains can not be considered enteropathogenic since they produced neither enterotoxins (STa, STb or LT1) nor cytotoxins (VT or CNF).

Epidemiological studies have shown that α-hemolysin production is correlated with *E.coli* that causes animal extraintestinal infection, mainly associated to CNF production (5) and human uropathogenic infection and sepsis (23). An association of α-hemolysin production with virulence of some *E.coli* strains was detected in experimental infections in laboratory animals. However, the role of α-hemolysin in human and animal enteric and extraintestinal infections is not clear yet. Also, very little is known on the role of cell-bound *E.coli* hemolysins, like β- and enterohemolysins, in bacterial virulence. However, no MMA *E.coli* strain of this study produced α-hemolysin or enterohemolysisn (E-Hly), demonstrating that the hemolysins are not a virulence factor in MMA syndrome. The gene carriers

of colicin V that also carry other genes responsible for pathogenicity (26, 27) would enable the strains to overcome the iron-limiting conditions that exist in the host body fluids by producing an iron acquisition system induced by the low levels of iron in these fluids.

The *E.coli* strains did not adhere to SK6, PK15 or C17 cells. In preliminary assays, these strains were also negative for HeLa cells (data not shown). We suggest that other cell lines be used to test this mechanism of pathogenicity.

Although *E.coli* is known to be one of the main bacteria associated to MMA syndrome (2, 32, 33, 39), it was not possible to define in this study the virulence profile of the MMA *E.coli* strains. We believe that many virulence factors contribute to their pathogenicity, which is undoubtedly multifactorial.

ACKNOWLEDGEMENTS

This study was supported by grant 502269/910 from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and grant 92/4297-0 from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

RESUMO

Fatores relacionados com virulência em amostras de *Escherichia coli* isoladas de porcas com síndrome de Mastites-Metrites-Agalactia (MMA).

Foram examinadas as características biológicas de 30 amostras de *Escherichia coli* isoladas de porcas com síndrome de Mastites-Metrites-Agalactia (MMA) que podem estar relacionadas com fatôres de virulência. Nenhuma amostra produziu enterotoxinas (LT1 ou STa), citotoxinas (Vts, CNF) ou hemolisinas (α-Hly e E-Hly). 25 amostras apresentaram-se resistentes ao soro normal de suíno, 17 produziram aerobactina e 14 produziram colicina V. Onze amostras expressaram fímbria tipo 1. Nenhuma amostra expressou fímbria P1 e F165. Não foi observada a capacidade de adesão a células HeLa e de rim de porcos (SK6, PK15, C17). Os resultados obtidos sugerem que a patogenicidade destas amostras de *E.coli* é multifatorial, requerendo estudos moleculares para a determinação dos marcadores de virulência.

Palavras-chave: *Escherichia coli*, Mastites-Metrites-Agalactia (MMA), suínos, fatores de virulência.

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Revista de Microbiologia (1997) 28:61-64
ISSN 0001-3714

COMPARATIVE "*IN VITRO*" STUDY ON THE SUSCEPTIBILITY AND EMERGENCE OF MUTANTS RESISTANT TO DANOFLOXACIN AND CIPROFLOXACIN AMONG *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE MASTITIS

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SHORT COMMUNICATION

ABSTRACT

The antimicrobial susceptibility of danofloxacin was compared *in vitro* with ciprofloxacin against mastitogenic *Staphylococcus aureus*. Danofloxacin was more active than ciprofloxacin, showing minimal inhibitory concentrations (MIC_{90}), minimal bactericidal concentrations (MBC_{90}) and MBC/MIC ratio lower than those of ciprofloxacin. Ciprofloxacin-resistant mutants occurred at higher frequencies ($\geq 10^{-6}$) than for danofloxacin (10^{-9}).

Key words: *Staphylococcus aureus,* danofloxacin, ciprofloxacin, susceptibility, resistant mutants.

DANOFLOXACIN is a new fluoroquinolone used exclusively in veterinary medicine against the most important pathogens with significance in animal infections(4,8,12). Like other fluoroquinolones, its pharmacokinetics has demonstrated a fast absorption after parenteral and oral administrations with a relatively long plasma half life and an excellent concentration in lung tissues. Thus, it has been used especially for the treatment of infectious respiratory diseases in cattle, poultry and swine (6,7). Current significant studies have also shown danofloxacin with a good *in vitro* activity against *Mycoplasma*, bacterial species, such as: *Pasteurella haemolytica, Pasteurella multocida, Haemophilus somnus, Escherichia coli* and other pathogens isolated from pulmonary infections of food-producing animals (2,5,17).

Only two mechanisms of resistance to quinolones have been identified until now: mutation in the target DNA gyrase and mutations which seem to decrease drug permeation (1,15,21). The single-step chromosome mutations occur at a low frequency (10^{-9}) in several bacterial species, and generally result in a low level resistance. However, high-level resistance following several mutations can be induced by serial exposures of bacteria to increasing concentrations of fluoroquinolones(21). Moreover, there is a concern that the widespread use of some fluoroquinolones may result in adverse clinical circumstances, as in the development of resistance during treatment(10,13).

Despite the extensive research and the relevant reports on the *in vitro* activity and on the emergence of fluoroquinolone resistance in Gram-negative

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bacteria(3,17,19), data on Gram-positive ones are scarce, especially in veterinary medicine. Thus, the objective of the this paper was to study comparatively the efficacy of danofloxacin and ciprofloxacin against strains of *Staphylococcus aureus* isolated from clinical bovine mastitis, as well as the emergence of spontaneous mutants resistant to fluoroquinolones.

One hundred and ninety-six *S. aureus* strains from milk samples collected from cows suffering from clinical mastitis on five dairy farms in São Paulo State, Brazil, were consecutively isolated from September 1991 to October 1995. Strains were identified by standard bacteriological methods (9) and, for further studies, one strain from each positive sample was chosen at random.

For minimal inhibitory concentration (MIC) testing, the antimicrobials in dry powder of known potencies were kindly supplied by Pfizer Co., (Danofloxacin -DAN) and by Bayer Co., (Ciprofloxacin-CIP). Stock solutions were freshly prepared according to instructions of the manufacturers and used within 12 h.

Comparative *in vitro* efficacy of DAN and CIP was assayed by the microdilution method(14). The antimicrobials were prepared in microtiter plates (Denka Seiken Co.) by serial twofold dilutions with concentrations ranging from 0.015 to 32 mg/l in Mueller Hinton Broth (Difco), and suspensions from log-phase bacterial cultures adjusted to a final concentration of 5×10^5 UFC/ml. After incubation at 37°C for 18 h, the MIC was read as the lowest concentration of the antimicrobial agent preventing visible growth. *Staphylococcus aureus* strains AI-87 [stable mutant resistant to DAN (8 mg/l)] and I-192 [stable mutant resistant to CIP (16 mg/l], isolated and characterized in our laboratory, were used as positive controls. Reference strain *Staphylococcus aureus* ATCC 29213 was used as negative control. MIC breakpoint of 4 mg/l was used following the criteria of Koneman *et al.*(9). MBC was determined as described by Stanholtzer *et al*(20). MBC was defined as the lowest concentration of antimicrobial agent that killed 99.9% of the original inoculum after a subsequent 18-24-hour incubation on Columbia Agar Base (Difco) plus 5% of defibrinated sheep blood.

Spontaneous mutants resistant to DAN and CIP were detected by plating 0.1 ml of an overnight culture (10^8 CFU/ml) onto Mueller Hinton Agar (Difco) plates containing the antimicrobial drug at a concentration of four and eight times the MIC. After incubation for 48 h at 37°C, CFU were determined and the frequency of spontaneous resistant mutants was calculated.

Table 1 shows the *in vitro* activity of the antimicrobials based on the MICs and MBCs. In regard to the MIC breakpoint, all isolates were defined as sensitive to both quinolones, although DAN showed to be slightly more active than CIP, with more than 90% of the strains being inhibited in the MIC range of 0.015 to 0.25 mg/l (MIC 90% - 0.18 mg/l), and killed in the MBC ranging from 0.015 to 1 mg/l (MBC_{90} - 0.23 mg/l). These data correlate with those of other investigators in Gram-negative bacteria (3,5,17) , but not with the antimicrobial profiles of some clinical isolates from human infections (1,10,11). On the other hand, the MBC/MIC ratios (Table 2) showed that the highest indices were obtained for CIP, with two strains showing ratios of 16: 1 and 32: 1, respectively. However, the distribution of DAN ratios was more

Table 1 - Comparative *in vitro* activities of Danofloxacin and Ciprofloxacin against 196 strains of mastitogenic *Staphylococcus aureus*

	MIC (mg/l)				MBC (mg/l)		
antimicrobial	breakpoint	range	MIC (a)	MIC (b)	Range	MBC (a)	MBC (b)
Danofloxacin	4	0.015-0.25	0.046	0.18	0.015-1	0.072	0.23
Ciprofloxacin	4	0.015-0.5	0.048	0.2	0.015-1	0.14	0.45

MIC (a) Minimal inhibitory concentration for 50% of strains
MIC (b) Minimal inhibitory concentration for 90% of strains
MBC (a) Minimal bactericidal concentration for 50% of strains
MBC (b) Minimal bactericidal concentration for 90% of strains

Table 2 - MBC/MIC ratio for 196 strains of mastitogenic *S. aureus*

antimicrobial	Number of strains (%) with the MBC/MIC ratio					
	1:1	2:1	4:1	8:1	16:1	32:1
Danofloxacin	143 (72.9)	28 (14.5%)	10 (5.1)	15 (7.5)	0	0
Ciprofloxacin	95 (48.5)	72 (36.8)	25 (12.7)	0	2 (1.0)	2 (1.0)

homogeneous, with 72.9% of the strains being inhibited and killed by the same drug concentration (ratio 1:1), whereas for CIP the ratio 1:1 was observed in 47.9% of the strains. For practical purposes, a significative difference between MIC and MBC may reveal bacterial tolerance and can be a major factor in the choice of an antimicrobial agent for therapeutic strategy. According to Sabath *et al* (18), the growth of a tolerant strain is inhibited at low-drug concentrations like a non-tolerant strain, but killing occurs at much higher concentrations than for a non-tolerant strain.

Furthermore, our observations on frequencies of spontaneous resistant mutants showed that mutation to DAN occurred only at 10^{-9}, whereas for CIP mutations occurred at frequencies ranging from 10^{-6} to $\leq 10^{-9}$. The frequencies of mutation for CIP were also comparable with those reported by Watanabe *et al* (21) and Piddock (16) as well as with the observations of Lopes *et al* (11) based on a study with multidrug resistant isolates of *S.aureus* from human infections.

In conclusion, DAN and CIP displayed an excellent antimicrobial activity against mastitogenic *Staphylococcus aureus*, with DAN exhibiting a better performance than CIP. Based on the significative inhibitory and bactericidal activities, low frequency of emergence of spontaneous resistant mutants and a possible favourable profile of its pharmacokinetics in the mamary gland, DAN may be indicated as a promising agent for the control of staphylococcal mastitis.

RESUMO

Estudo comparativo "*in vitro*" da suscetibilidade e emergência de mutantes resistentes a danofloxacina e a ciprofloxacina entre linhagens de *Staphylococcus aureus* isoladas de mastite bovina

A atividade antimicrobiana da danofloxacina foi avaliada comparativamente "*in vitro*" com a da ciprofloxacina frente ao *Staphylococcus aureus* mastitogênico. A danofloxacina foi mais ativa com CIM_{90}, CBM_{90} e índice CBM/CIM inferiores aos da ciprofloxacina. A ocorrência de mutantes resistentes à ciprofloxacina e à danofloxacina foi de $\geq 10^{-6}$ e de 10^{-9}, respectivamente.

Palavras-chave: *Staphylococcus aureus*, danofloxacina, ciprofloxacina, suscetibilidade, mutantes resistentes.

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Revista de Microbiologia (1997) 28:65-67
ISSN 0001-3714

INDUCTION OF PHYSICAL PARAMORPHOGENESIS IN *ASPERGILLUS* SP

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SHORT COMMUNICATION

ABSTRACT

A simple and easily reproducible method to obtain little fungical pellets from mycelial fractionization, completely under control with respect to development, size and homogeneity without genetic compromise, is described. The method has been successully employed with *Aspergillus niger* and also showed similar results with *Aspergillus oryzae*.

Key words: *Aspergillus niger, A. oryzae*, paramorphogenesis, filamentous fungi, adsorption.

The paramorphogenic method was originally developed for adsorptive interaction studies among filamentous fungi and industrial dyes. However nothing prevents its utilization for other purposes, such as in bioreactors for the production of enzymes, antibiotics, organic acids, metabolic products, etc. (4) This method aims to obtain inocula and mycelial pellets, which are homogeneous, small-sized, asseptic and easy to handle, offering greater and more uniform active surface. In this way, a morphologically different fungic form is obtained. The reproductive potential is not altered, since the alteration is caused by the manner the filamentous fungus grows, which is "rolling up", due to the physical dynamics of the rotary table. The paramorphogenic method was compared with the traditional method of inoculation of fungical spores, confirming the greater homogeneity of the pellets' size in the biomass production.

Aspergillus niger culture, maintained in 2% malt extract at 4°C, was transferred to 250 ml erlenmeyers containing 100 ml of growth mineral medium for *Aspergillus* sp (2) and different quantities (1, 5, 10, 15 and 20 g) of glass cylinders (6 mm diameter and 2 mm thickness). The flasks were put in a shaker at 250 rpm and 30°C and cultivated for 24 and 48 hours. This paramorphogenic method was compared with the spore inoculation method, using sporulated cultures of *A. niger* obtained in 2% malt extract medium plus 0.1% Tween 80 (spore suspension). The contents of each erlenmeyer was inoculated with 1 ml of this spore suspension.

Samples obtained through the paramorphogenic method were heated and dried at 105°C in a stove and slides of these cultures were photographed through a photomicroscope with amplification 12.5 x 3.2 x 4.0 . The cultivation type presenting the best cultural aspect and the best reproducibility was recorded. The best result was obtained with pre-inoculum obtained in erlenmeyers containing 10g of glass cylinders (Table 1) . This pre-inoculum presented large amounts of

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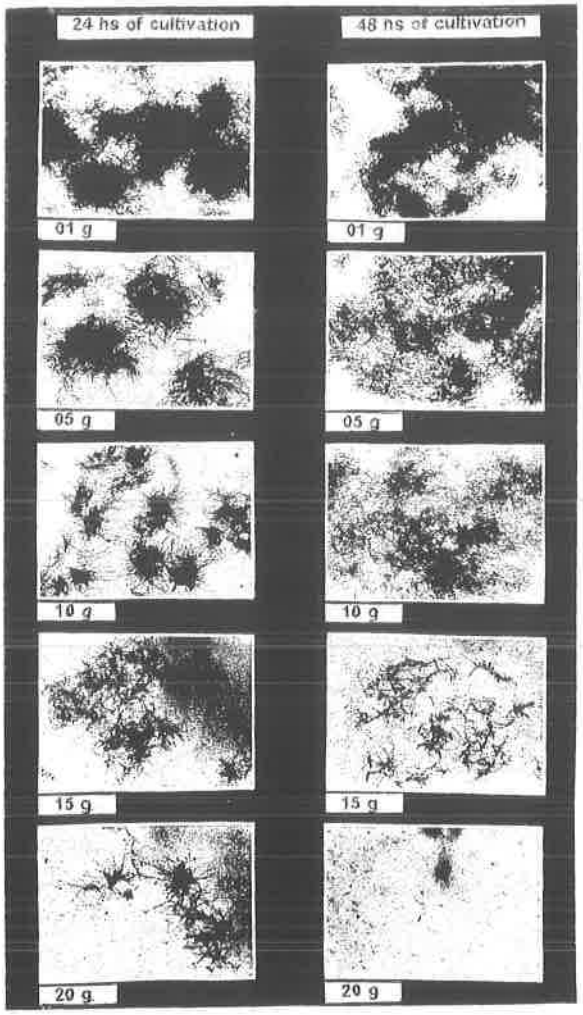


Figure 1: Cultures of *Aspergillus niger* obtained after 24 and 48 h at 29±1°C in shaker (250 rpm) using different amounts of glass cylinders. Anatomic X film, 32 ASA. Amplification: 12,5 x 3,2 x 4,0.

mycelial pellets of approximately 340 mm after 24 h of cultivation (Figure 1, Table 1), being the hyphae looser than in other samples. Other quantities of glass cylinders were ineffectual to lacerate the hyphae: 15g or 20g interfered with growth and reprodution, and 1g and 5g were insufficient to cause an adequate cellular fractionization.

From the pre-inoculum obtained with 10 g of glass cylinders after 24 h of cultivation, aliquots of 1, 2 and 4 ml were inoculated into erlenmeyers containing 100 ml of growth medium for *Aspergillus* sp and placed on a shaker at 30°C and 250 rpm. Samples were taken after 8, 16, 24, 32, 40 and 48 h of cultivation to obtain dry weight biomass (at 105°C). The results were

Table 1: Biomass obtained after 24 and 48 h of *Aspergillus niger* cultivation in shaker, at 29±1°C, with different amounts of glass cylinders.

Time of cultivation (h) / glass cylinders	1 g	5 g	10 g	15 g	20 g
24	0,3905	0,2240	0,1220	0,0967	0,0560
48	0,5245	0,3957	0,3625	0,1877	0,1866

Table 2: Statistical evaluation of the homogeneity of the amount of biomass obtained by the spore inoculation and paramorphogenic methods in *Aspergillus niger* after 24 and 48 h of cultivation in shaker at 29±1°C, using 1 ml of inoculum.

Method	Spore Inoculation		Paramorphogenic	
	24 h	48 h	24 h	48 h
Patterns score	2,0705	4,3785	3,7285	5,1510
Average (g of dry weight/100 ml)	0,2070	0,4378	0,3728	0,5151
Standard deviation	0,07478	0,08355	0,01506	0,00495
C.I. (Confidence Interval)	0,2070± 0,1690	0,4378± 0,1888	0,3728± 0,0340	0,5151± 0,0111
Variance	0,00559	0,00698	0,00022	0,00002

F of 24 h: 25,409; F of 48 h: 284,897.

statistically evaluated by the F test (3)$F = S^2_1 \div S^2_2$ where:

F: critical value of sample to be compared with F* (0,025), with freedom degree 9,9: 4,03.

S^2_1: treatment variance

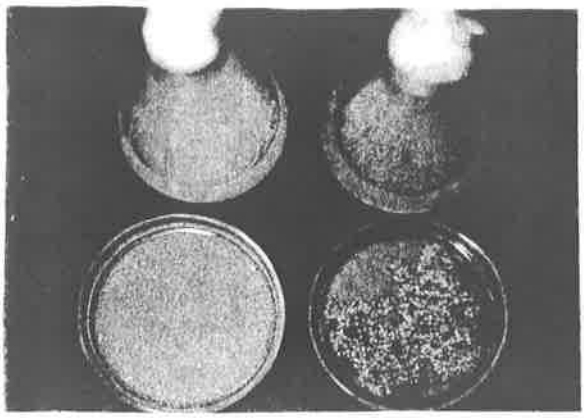
S^2_2: residual variance

Confidence Interval : C.I.: $X \pm t.\ s$, where X: average of the samples, t: (Student), with 9 degrees of freedom and confidence level of 95%, and s: standard deviation of samples.

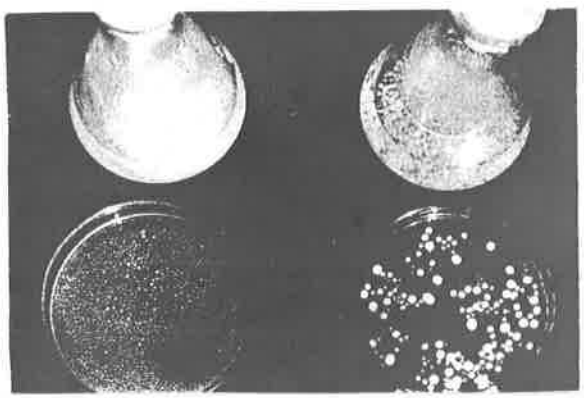
The dry weights of the biomass samples were inversely proportional to the amount of glass cylinders (Table 1). Thus, the possibility of obtaining adequate fungal growth decreases with 15 and 20 g of cylinders.

The best amount of inoculum was 4 ml, and the end of the linear growth was achieved after 24 h of cultivation, resulting in pellets around 0.5 and 1 mm in diameter and greater amount of biomass. Smaller amounts of inoculum resulted in less biomass, with larger pellets (2-2,5 mm for 1 ml / inoculum).

The comparison between paramorphogenic and spore inoculation methods showed that the pellets obtained through the first method were uniform in size (0.5 - 1 mm), while in the spore inoculation method the diameter of the pellets varied from 2 to 8 mm



PARAMORPHOGENESIS	SPORE INOCULATION
A. niger - 24 h of cultivation	*A. niger* - 24 h of cultivation



PARAMORPHOGENESIS	SPORE INOCULATION
A. niger - 48 h of cultivation	*A. niger* - 48 h of cultivation

Figure 2: Comparison of *A. niger* mycelia fragmentation obtained by paramorphogenic and spore inoculation methods after 24 and 48 h of cultivation time, at 291°C, in shaker (250 rpm).

(Fig.2), suggesting an irregular growth, probably due to differences in latence and germination time of each spore. The paramorphogenic method also resulted in greater biomass content (Table 2) and greater homogeneity in pellet formation. Fisher test showed significant differences in F* values for 24 and 48 h, in a limit of 2.5%.

It was therefore possible to conclude that the paramorphogenic method results in small, uniform and standardized mycelial pellets, asseptic and easy to handle, generating greater biomass content. The variances of the samples prepared through this method were significantly lower than those obtained through the inoculation spore method.

RESUMO

Indução de paramorfogênese física em *Aspergillus* sp.

Um método simples e de fácil reprodutibilidade para obtenção de pequenos pellets fúngicos a partir de fracionamento micelial, perfeitamente controlável quanto ao seu desenvolvimento, tamanho e homogeneidade, sem comprometimento genético, é apresentado. Ele tem sido empregado com sucesso em *Aspergillus niger*, apresentando resultados semelhantes também em *Aspergillus oryzae*.

Palavras-chave: *Aspergillus niger*, *A. oryzae*, paramorfogênese, fungos filamentosos, adsorção.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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