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Objetivos

A Sociedade Brasileira de Microbiologia, fundada em 28 de setembro de 1956, é sociedade civil, sem fins lucrativos, dedicada a agremiar microbiologistas brasileiros; a promover o desenvolvimento da microbiologia através do estímulo à pesquisa científica e suas aplicações, melhorar as qualificações profissionais dos microbiologistas; e manter intercâmbio, a cada dois anos, na última semana do mês de julho, o Congresso Brasileiro de Microbiologia e edita a Revista de Microbiologia, com distribuição trimestral. Para informações sobre a SBM, escrever para qualquer membro da Diretoria.

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EDITORIAL

Prezados colegas

A Sociedade Brasileira de Microbiologia e os responsáveis pela produção da Revista de Microbiologia tem envidado todos os esforços possíveis no sentido de melhorar sua qualidade a cada número.

Entre esses esforços, destacam-se a contratação de uma tradutora profissional, Doutora em Ciências, para a revisão do Inglês dos trabalhos aceitos para publicação, e o aumento do grau de informatização da revista, através da utilização de disquetes para encaminhamento dos trabalhos para revisão e impressão.

A partir do próximo número será introduzido o cargo de Editor Seccional, isto é, cada área de especialização ou áreas mais afins será(ão) coordenada(s) por um Editor especialista nessa(s) área(s). Isto possibilitará melhor apreciação dos trabalhos submetidos à publicação, pois caberá ao Editor Seccional a seleção inicial, a definição dos revisores, a coordenação da revisão e, finalmente, quando o artigo houver passado por todas as fases da avaliação, o encaminhamento à Diretoria da Revista para a revisão do Inglês. A Diretoria reencaminhará os trabalhos para o(s) autor(es) para que sejam feitas as modificações necessárias, e após a devida devolução dos trabalhos corrigidos, em disquete, para a Diretoria, esses serão encaminhados para a gráfica para a impressão. A primeira prova vinda da gráfica será enviada ao(s) autor(es) para conferência final antes da impressão definitiva.

Os nomes e endereços dos Editores Seccionais serão publicados a partir do próximo número, devendo o(s) autor(es) selecionar o Editor mais afim com sua especialidade e enviar seu trabalho diretamente ao mesmo.

Um novo Conselho Editorial será também definido, que terá a função de avaliar a Revista e definir a filosofia de publicações da mesma, bem como servir de árbrito final caso o(s) autor(es) cujos trabalhos forem recusados recorrer(em). O número de participantes do novo Conselho Editorial será menor que o atual pois muitos dos colegas que constam da lista atualmente pertencem ao corpo de revisores e, como essa função é confidencial, passarão a não constar da lista a ser publicada nos próximos números. O novo Corpo Editorial (Diretoria, Assistentes, Conselho, Editores Seccionais e revisores) terá duração de dois anos e será, portanto, revisto em 1997, podendo ser modificado se necessário.

A aceitação da Revista de Microbiologia tem melhorado bastante e temos recebido trabalhos de bom nível, o que forçosamente terá reflexos no seu índice de impacto.

Apesar de todos os esforços, ainda há varios problemas a serem sanados visando a melhoria da Revista de Microbiologia. É preciso que o(s) autor(es) entenda(m) e sigam as orientações dadas pela Revista quanto à forma, originalidade, atualidade do tema, redação adequada e clara dos trabalhos a serem submetidos a publicação, bem como efetuem a conferencia minuciosa do texto para detecção dos erros de redação e impressão. Os gráficos, figuras e fotos, quando extremamente necessários, deverão ser bem nítidos e corretos e com títulos adequados e legíveis. As referências bibliográficas deverão ser preparadas de acordo com as normas da Revista.

É preciso também que os revisores cumpram efetivamente seu papel, efetuando uma avaliação profunda e detalhada do trabalho, avaliando-o quanto à sua originalidade e atualidade, e, inclusive, quanto à forma, qualidade da redação, descrição de material e métodos, apresentação de resultados, ilustrações, referencias bibliográficas que devem ser pertinentes, atualizadas e apresentadas de acordo com as normas.

Outro item importante é quanto ao tempo gasto pelos árbritos para a revisão e pelo(s) autor(es) para devolução dos trabalhos após a reformulação do texto, a revisão do Inglês e a correção final. Os prazos dados não tem sido obedecidos prejudicando a Revista por atrasar a sua publicação. A administração da Revista passará a usar suas atribuições desqualificando os trabalhos cujos autores não atenderem os prazos dados e desconsiderando as avaliações cujos árbritos não forem pontuais nas respostas.

Outro aspecto importante que merece atenção é a grande quantidade de erros como, por exemplo, nomenclatura, não atendimento às normas para referências bibliográficas, gráficos e fotos ilegíveis ou com apenas uma cópia e tabelas com erros. A redação em Inglês deve também ser melhorada e, apesar de termos uma revisora do Inglês, o manuscrito deve ser redigido com muito cuidado, recomendando-se que os autores submetam seus trabalhos a um especialista do idioma, para que o mesmo chegue à Revista com a maior qualidade possível. Ocorre que, muitas vezes, o textos são tão mal preparados que se tornam incompreensíveis, prejudicando o trabalho da tradutora e, consequentemente, a qualidade da Revista. Outro ponto que gostaríamos de salientar é que alguns colegas tem questionado o "por que" da publicação em Inglês. Queiramos ou não, o Inglês tornou-se a praticamente o idioma científico. Somos cobrados por nossas Instituições e Agencias de Fomento a publicar nesse idioma. Sómente dessa maneira a produção científica brasileira será reconhecida no Exterior. A Revista de Microbiologia é publicada desde 1970, e é uma das raras publicações científicas nacionais que mantém sua continuidade e periodicidade; tem melhorado seu nível contínuamente graças à colaboração dos senhores autores e, portanto, precisa e deve ser publicada em Inglês. Existem no país diversas outras revistas que aceitam publicações em Português, portanto, cabe à comunidade científica a escolha daquela mais conveniente.

A partir do próximo número, a Revista de Microbiologia transferirá para o(s) autor(es) a responsabilidade de efetuarem a correção final de seu trabalho. Nessa fase, o(s) autor(es) receberá(ão) uma cópia da primeira prova vinda da gráfica, devendo então, conferir toda a impressão palavra por palavra, letra por letra, número por número. Nessa fase, não poderão ser mais aceitas alterações no texto. O prazo para devolução da prova corrigida é de apenas 5 dias, pois, para que a Revista continue indexada no "Current Contents", a pontualidade e a periodicidade constituem itens importantes na avaliação.

Temos também sido cobrados pela melhoria do "índice de impacto" da Revista de Microbiologia. Esse índice demonstra a importancia dos conhecimentos gerados por um artigo científico, isto é, o impacto de determinada pesquisa na sua área de conhecimento. Esse índice baseia-se no número de citações de um determinado artigo em artigos redigidos por outros autores. O índice de impacto de uma revista é melhorado à medida que aumenta o número de citações dos trabalhos nela publicados.

O índice de impacto e o índice de citação das revistas científicas são publicados no "Science Citation Index", e esses índices, quer sejam aceitos ou não pela comunidade científica, são um dos fatores mensuráveis utilizados para verificar como se comportam as diferentes áres da ciência de um país e para a definicão de prioridades para seu apoio. Esses índices dependem, portanto, unica e exclusivamente dos autores, isto é, da comunidade cientíifica. O mesmo vale para o seu indexador, pois se os trabalhos não forem bons, o periódico corre o risco de não ser indexado.

Portanto, de nada adianta termos um Conselho Editorial composto por cientistas de renome internacional, assim como de nada adiantam os esforços dos editores, se os trabalhos científicos não tiverem uma proposta que leve ao avanço dos conhecimentos em uma determinada área, baseados em conceitos e metodologia adequados e atualizados.

Houve, durante um certo tempo, uma falta de apoio de algumas agencias de fomento que, orientadas por alguns assessores, não consideravam as publicações na Revista de Microbiologia como produção científica. Muitas dessas instituições não

sabiam que a Revista de Microbiologia era indexada no Current Contents. Nessa época, muitos bons autores deixaram de publicar na nossa Revista e houve uma queda no índice de impacto. Isto foi esclarecido, as publicações estão melhorando e o índice já está subindo. Subirá ainda mais quando os colegas adotarem o hábito saudável de citar trabalhos de colegas nacionais, inclusive dos artigos que os estimularam em suas pesquisas.

Outra informação importante: embora não se pretenda mudar o nome da Revista de Microbiologia, a partir de 1995, seu nome será citado também em Inglês (Journal of the Brazilian Society for Microbiology). Como a Revista é adquirida por várias bibliotecas internacionais, é importante que cientistas de outros idiomas a reconheçam.

Outro aspecto importante é que a descentralização da Revista, através da implementação dos Editores Seccionais, possibilitará que membros de outros Estados da Federação e de diferentes instituições tenham uma contribuição efetiva no corpo editorial da Revista de Microbiologia. Dessa forma, ficará claro que, apesar do Departamento de Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo haver gentilmente cedido uma sala para a Revista de Microbiologia e de vários professores desse Departamento estarem trabalhando na sua produção, a Revista de Microbiologia pertence, efetivamente, à Sociedade Brasileira de Microbiologia, e portanto, à toda a comunidade científica nacional, e não ao Departamento de Microbiologia da USP.

Temos certeza que essas inovações, somadas aos esforços do Prof. Dr. Luiz Rachid Trabulsi, criador da Revista de Microbiologia, e de vários editores, principalmente o Prof. Dr. Flávio Alterthum e Prof. Dr. Walderez Gambale, que durante muitos anos dirigiram a Revista, e à contribuição de todos os nossos autores e colaboradores, farão com que a Revista de Microbiologia dê um salto de qualidade, atingindo efetivamente o nível que almejamos entre as publicações internacionais. Contamos para isso com a colaboração de todos, submetendo seus melhores trabalhos para publicação em nossa Revista, divulgando-a e defendendo-a quando necessário.

Profa. Dra. Maria Therezinha Martins
Presidente da SBM
Diretora da Revista de Microbiologia

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MINISATELLITE PROBES IN YEAST DNA FINGERPRINTING

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SUMMARY

The minisatellite sequences 33.6, 33.15, M13 and R18.1 were used as probes for yeast DNA fingerprinting. Probe R18.1, derived from a cattle genomic bank, showed strong hybridization, producing complex and informative DNA fingerprinting profiles both in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*. High polymorphism was observed in intra and interespecific comparisons, allowing the identification of all the strains, even those closely related.

Key words: DNA fingerprinting; yeasts; repetitive DNA; genetic distances

INTRODUCTION

Yeast classification is traditionally based on morphological and physiological criteria. However, these phenotypic traits commonly fail to discriminate among strains within a single species. As most industrial yeasts used in ethanolic fermentation processes belong to the *Saccharomyces* group, they are not readily identified by the classical methods; this constitutes an important challenge to yeast breeders and yeast producers, who seek to give the user a quality product with a predictable influence on the fermentation process and final manufacturing good. Several alternative methods, such as total proteins (12) and isoenzymatic (10) electrophoretic patterns, pulse-field electrophoresis for karyotype comparisons (2) and analysis of DNA polymorphisms (2, 6, 8, 13), have been recently used with a variable success in the identification and characterization of yeast lines.

One of the most efficient tools for the identification of individuals and the investigation of genetic variability is DNA fingerprinting analysis based on the hybridization of suitable probes to hypervariable, dispersed repetitive DNA sequences (minisatellites). DNA fingerprinting analysis provides highly polymorphic profiles with great potential as genetic marker of several eukariotic organisms such as animals, plant and fungi (1). Yeast DNA fingerprinting profiles have been obtained using the transposable element Ty sequence, the telomeric Y' element and poly (GT) microsatellites (2, 6, 13), allowing the identification of several strains of baker's, brewing and wine yeasts.

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In the present paper we report that some minisatellite probes, especially probe R18.1 (3) derived from a cattle genomic bank , generate complex DNA fingerprinting patterns that allow the identification of lines within the *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* yeast species.

MATERIALS AND METHODS

Tests were performed with *S. cerevisiae* strains J132b, X2180-1B and MXV (X2180-1B x M304-2C). The haploid strain KM9 and the diploid MM1 (KM9 x KM21) belong to the *K. marxianus* species. Total DNA was prepared from protoplasts by the CTAB method (7) and 5 µg aliquots were digested with EcoRI, BamHI, PstI, HindIII and DraI. The electrophoretic separation of DNA fragments was made in 20x20 cm 0.8% agarose gels, using TBE buffer (0.089 M Tris-Borate, pH8.3 and 0.025M Disodium EDTA). Southern blotting and hybridization were performed as described by Tzuri et al. (11). Probes used for hybridization were as follows: 33.6 and 33.15 (4), M13 (9) and R18.1 (3). The profiles were visually compared and their relation expressed as bandsharing frequences (BS) calculated according to the following formula (5): BS=2 (Nab) / (Na+Nb), where Nab is the number of bands shared by lines a and b; Na and Nb are the total number of bands observed in lines a and b, respectively.

RESULTS AND DISCUSSION

Due to the low number of bands and the poor hybridization obtained, probes 33.6, 33.15 and M13 were considered innadequate for yeast DNA fingerprinting analysis. Informative DNA fingerprinting patterns were obtained using probe R18.1. Complex profiles with 14 to 26 bands were obtained (figure 1). The number of bands in each profile was dependent on the enzyme and genotype. Considering the sequence of probe R18.1 (3), the present results confirm the presence of several poly (GT/CA) sequences distributed within the *Saccharomyces* and *Kluyveromyces* genomes, as previously described by Walmsley *et al.* (13) using microsatellite poly (GT) probes. The strong hybridization against *Saccharomyces* and *Kluyveromyces*

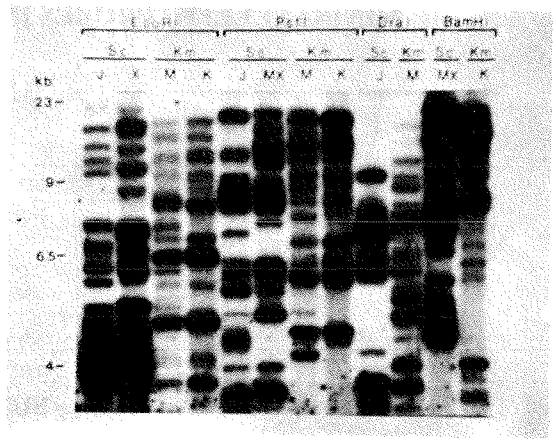

FIGURE 1 - DNA fingerprinting profiles of yeast using probe R18.1 and the restriction enzymes EcoRI, PstI, DraI and BamHI. Lines are (J) J132b, (X) X2180-1B and (MX) MXI of *Saccharomyces cerevisae* (Sc) and (K) KM9, (M) MMI of *Kluyveromyces marxianus* (Km).

fragments suggest a high homology between probe R18.1 and yeast sequences. The qualitative (presence/absence) comparison of the profiles allowed the identification of all the lines analysed, discriminating even between those closely related, such as KM9 and MM1. The number of discriminating bands that differed (presence/absence) in each comparison ranged from 10 to 32, thus providing a high number of potential genetic markers for strain identification and genetic analysis. Very low bandsharing frequencies of 0.06 to 0.16 were obtained for the interespecific comparisons between *Saccharomyces* and *Kluyveromyces* strains, whereas frequencies of 0.40 to 0.44 were observed among *Saccharomyces* strains and of 0.76 to 0.82 when comparing KM9 and MM1 of *K. marxianus.* Such frequencies are consistent with those expected by parental relations. All the bands in KM9 profiles were also presented by the diploid hybrid MM1, an indication of a conservative and heritable system. The bands that were only present in MM1 can be attributed to the other parental line, providing a means to predict the DFP pattern of the unknown parents.

DNA fingerprinting is expected to be a useful tool for the identification of strains, evaluation of genetic relationships and patenting of industrial yeasts, since it provides information on a large number of loci and is not affected by environmental factors.

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RESUMO

Identificação de leveduras pelo uso de sondas minisatelites

As sequências 33.6, 33.15, M13 e R18.1 foram utilizadas como sondas para a análise do parentesco de linhagens de leveduras. A sonda R18.1, originária de um banco genômico bovino mostrou intensa hibridização, produzindo perfis com alto grau de polimorfismo do DNA em *Saccharomyces cerevisiae* e *Kluyveromyces marxianus*. Nas comparações intra e interespecíficas, os perfis polimórficos permitiram identificação de todas as linhagens, mesmo aquelas altamente relacionadas.

Palavras-chave: DNA "fingerprinting"; leveduras; DNA repetitivo; distancias genéticas.

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CATIONIC INTERACTIONS IN THE LIPOPOLYSACCHARIDE OF *DESULFOVIBRIO VULGARIS*

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SUMMARY

High performance liquid chromatography (HPLC), energy-dispersive X-ray analysis and sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) were used to investigate the interactions between various cations and the lipopolysaccharide (LPS) of *Desulfovibrio vulgaris*. The LPS was shown to have different affinities for various divalent ions.

Electrodialysis removed a substantial amount of the iron(II) ions bound to LPS and caused an increase in calcium ions. Differences in the structure of LPS isolated from iron-rich and iron-poor cells demonstrated by HPLC and SDS-PAGE analyses indicated that iron(II) may have a role in stabilising sugar residues in the LPS molecule. This role is specific to iron (II), since increased calcium levels in iron-poor LPS do not maintain the structure. Sodium and potassium ions, however, appeared to be interchangeable in their binding to LPS.

The results provide additional evidence for the suggested role of *D. vulgaris* LPS in the uptake of iron(II) from the environment.

Key words: sulphate-reducing bacteria, ion-binding, *Desulfovibrio*, lipopolysaccharide.

INTRODUCTION

Desulfovibrio vulgaris is a Gram-negative anaerobic sulphate-reducing bacterium. It has a high requirement for iron for growth (14) and is abundant in many anaerobic environments, where it is often found in association with ferrous metal surfaces. The sulphate-reducing bacteria (SRB) in general are economically important as they can cause the corrosion of metal structures (8). Although several theories have been proposed to explain their mode of action in the anaerobic corrosion of iron (see references 10 and 11 for reviews), little work has been done on the interactions between iron surfaces and SRB cells, a process which is important in determining the rate of corrosion (9).

It is known that lipopolysaccharide (LPS) in the outer membrane (OM) of Gram-negative bacteria interacts with cations. This is important for OM assembly (15). Leive et al. (12) have demonstrated that ethylene diamine tetracetic acid (EDTA) releases LPS and proteins from the OM of some Gram-negative bacteria and that the mode of interaction between LPS and metal ions influences the yield of LPS released by EDTA treatment of whole cells.

The studies of Bradley et al. (4) suggest that iron(II) ions are important in the stabilisation "in vivo" of the OM of *Desulfovibrio vulgaris*

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(Woolwich). This could explain, in part, the requirement of these cells for iron(II) and their affinity, therefore, for iron-rich environments such as ferrous metal surfaces. The LPS of *Salmonella typhimurium* has both high and low affinity binding sites for magnesium and calcium (15) and contains multiple potential binding sites, whilst in *D. vulgaris* LPS has been shown to contain specific iron (II) binding sites(3).

The studies reported here were undertaken to estabilish the interactions between extracted LPS from *D. vulgaris* and various cations, in particular iron(II).

MATERIALS AND METHODS

Bacterial cultures

Desulfovibrio vulgaris (Woolwich) (NCIMB 8457) was grown at 30°C in Postgate's medium C (14) modified by the omission of citrate and ferrous sulphate for iron-poor (Fe-) cells and in Medium C plus a mild steel coupon for iron-rich (Fe+) cells. Inoculations were made from stock cultures grown in identical media. Iron levels in these media have been quoted previously (2).

LPS extraction

This was achieved by the hot phenol/water method of De Pamphilis (7) using cell envelopes as starting material. Cell envelopes were prepared as described previously (2).

Electrodialysis

Three to 5 mg/ml LPS samples in 2ml volumes were suspended within dialysis tubing (Visking size 1-8) in an electrophoresis tank with a circulating cooled water supply. The apparatus was run at constant voltage for 4-4.5h. The process was monitored by the change in current, which fell from 12-20mA to 4-5mA during the procedure.

X-ray dispersive microanalysis (EDAX)

LPS extracted from cells grown under different conditions of iron availability was manually deposited on to the surface of double-sided adhesive tape-coated aluminium stubs. The stubs were then carbon-coated to enhance the surface conductivity of the sample. The samples were analysed in a scanning electron microscope (Super Mini SEM, International Instruments) equipped with an energy-dispersive X-ray analyser (Lewell Electronics Ltd., UK) and Princeton gamma-Tech system 4 computer. The samples were scanned at an acceleration voltage of 15kv and a magnification of 1000. The beam current used for the analysis was between 5 and 6nA and the working distance was 23mm. X-rays were collected for 100 seconds. Three replicate analyses were performed on each sample and the counts for each element expressed as a percentage of the total counts for all elements present.

High performance liquid chromatography (HPLC) for sugar analysis

Sugars were released from LPS by acid hydrolysis with 0.5N hydrochloric acid in vacuum at 100°C for 4h. Such strong acid hydrolysis has been shown to be necessary to release sugars from *D. vulgaris* LPS, probably because of the lack of 3-deoxy-ketoaldonic acid in this molecule (1). The filtered hydrolysate was freeze dried and dissolved in 50 µl of column mobile phase (acetonitrile:water, 80:20 v/v) prior to analysis on a Waters HPLC system incorporating a U6K injector, 6000A solvent delivery system and a 401 refractive index indicator, using a Waters Bondapak aminocolumn.

Sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) of extracted LPS

LPS was run on 15% polyacrilamide gels prepared as previously described (2) and stained by the silver staining method of Morrisey (13).

RESULTS AND DISCUSSION

The results obtained from EDAX analysis of LPS are presented in Table 1. Major differences in ionic content between the Fe- and Fe+ LPS are found for the elements iron and calcium. These differences are not eradicated by electrodialysis. As can be seen from the results, electrodialysis removes only some iron. It is reported that cations tightly associated with *Escherichia coli* LPS cannot be removed by electrodialysis (5) and the data presented here indicate that such tight interactions occur between LPS and the detected metallic ions in *D. vulgaris*. The amount of iron detected in Fe+ LPS is greater than that in Fe-, as expected. This is reversed with respect to calcium

TABLE 1 - Percentage levels of iron, calcium, magnesium, sodium and potassium detected in iron-poor, iron-rich and electrodialysed iron-rich lipopolysaccharide samples from *D. vulgaris*. Values represent each ionic species relative to the total ions detected.

Element	% Present in Lipopolysaccharide		
	Fe+	Fe+(ED)	Fe-
Fe	21.0 3.0	4.7 1.8	4.3 0.1
Ca	12.7 0.5	28.8 2.1	18.7 4.1
Mg	3.5 0.4	3.2 0.3	4.2 1.1
Na	3.0 0.2	5.2 1.3	3.4 0.2
K	12.0 1.0	10.0 3.5	12.9 0.9

Fe+ = iron-rich
Fe- = iron-poor
ED = electrodialysed

levels, which are higher in Fe- LPS, but not for magnesium ions, whose levels are not significantly different in any extracted LPS. It seems that magnesium and calcium bind to different anionic sites in the LPS moiety. Magnesium and calcium are critical in the stabilisation of LPS within the OM of *E. coli* (6) and could function similarly in*D. vulgaris* LPS.

When iron is removed by electrodialysis from Fe+ LPS is an increase in the quantity of calcium (Table 1). Calcium may act, at least in part, as a substitute for iron in the neutralisation of charges at the anionic loci. There is only a small difference in the ionic radii of these two ions (calcium = 0.997A; iron(II) = 0.74A). The incomplete removal of iron from Fe+ LPS by electrodialysis (Table 1) suggests that of the iron detected in this sample, a small proportion is tightly bound. The vast majority, however, is loosely associated with the LPS and is removed by electrodialysis. The tightly bound iron ions could act as a focus for the accumulation of iron(II) ions from the environments prior to their uptake by the cells. This may occur in a two-step interaction. First there is a stoichiometric interaction between iron(II) ions and available anionic sites in the LPS. Secondly these bound ions act as the nucleus for accumulation of more iron(11) ions above the stoichimetric level.

The more tightly bound iron probably serves to maintain the integrity of the LPS molecule within the OM. This role is suggested by the results of SDS-PAGE analysis of *D. vulgaris* LPS (Figure 1), where an extra band (Relative mobility 0.58-0.64) is found in Fe- LPS. Additionally, the fastest moving band in Fe- LPS (Relative mobility 0.73-0.81) is wider than that in Fe+ LPS, suggesting an increase in number and diversity of shorter LPS

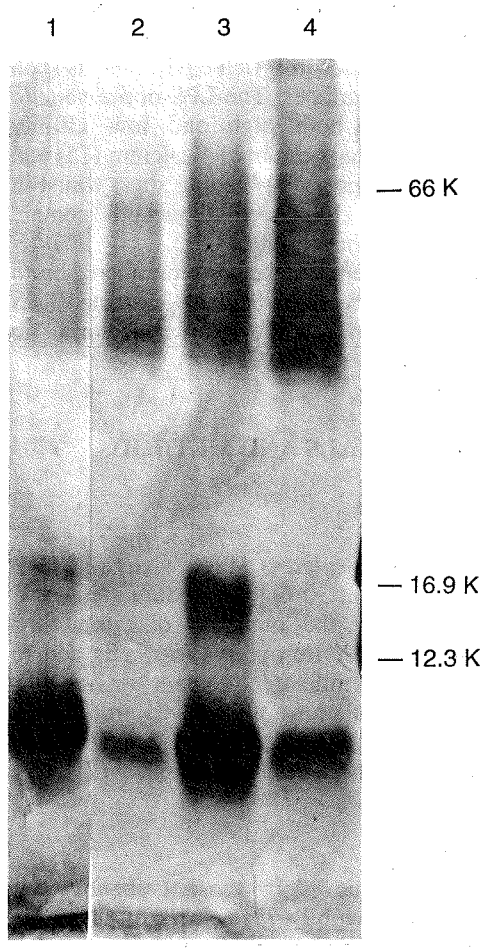

FIGURE 1 - SDS-PAGE of electrodialysed and non-electrodialysed lipopolysaccharide extracted from *D. vulgaris* grown in iron-rich or iron-poor conditions. Gel stained by the silver method (13).
Lane 1: Electrodialysed Fe- LPS
Lane 2: Electrodialysed Fe+ LPS
Lane 3: Fe- LPS
Lane 4: Fe+ LPS
Numbers on right hand side indicate positions and molecular weights of protein markers.

molecules due to degradation of LPS in the absence of iron. When Fe- LPS is electrodialysed prior to SDS-PAGE, there is a change in the electrophoretic pattern (Figure 1), showing a higher band intensity in the fast moving band and a decrease in the high molecular weight (slow moving) band. No such change is observed in Fe+ LPS on electrodialysis. Iron may, therefore, have a role in maintaining the integrity of LPS, the iron remaining bound to Fe+ LPS after electrodialysis (Table 1) being sufficient to maintain the polysaccharide chain lengths.

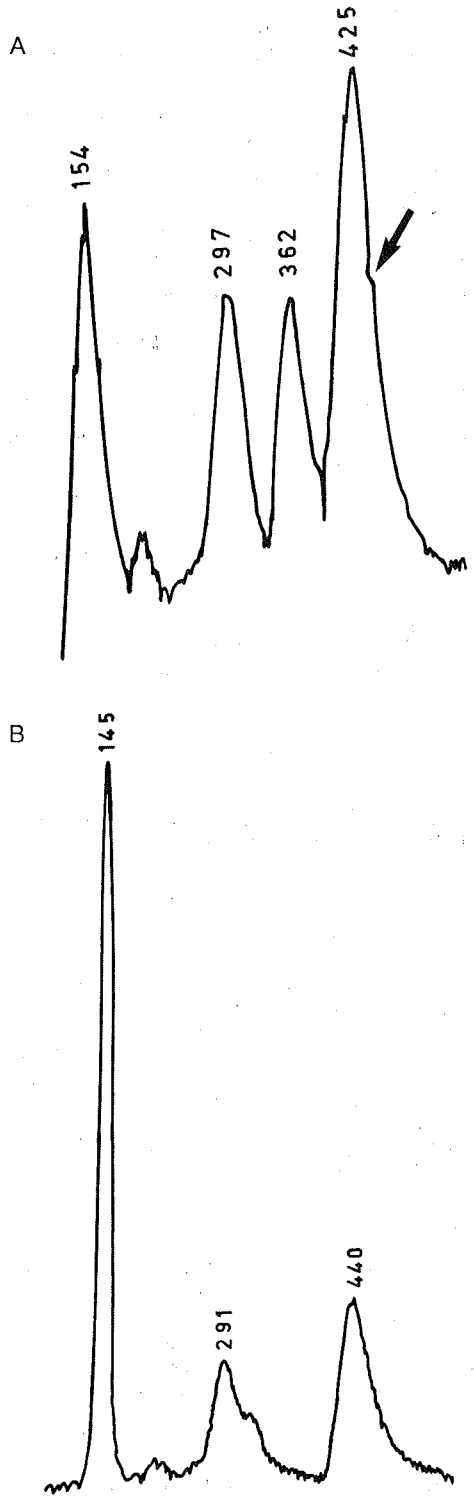

FIGURE 2 - HPLC sugar analysis of lipopolysaccharide extracted from *D. vulgaris* grown in iron-rich (A) or iron-poor (B) conditions. Numbers indicate retention times in seconds.

Calcium obviously cannot replace Fe(II) ions in this function, as the elevation in its level in Fe- LPS does not enable chain length to be maintained. It has previously been shown that calcium does not protect *D. vulgaris* LPS from the effect of EDTA treatment (4) and this present result confirms the specificity of the *D. vulgaris* LPS-Fe(II) interaction.

When the results of HPLC analysis of sugars released from LPS are examined (Figures 2 & 3), it is seen that there is a difference in the pattern between Fe+ and Fe- LPS samples. However, the electrodialysed Fe+ LPS chromatogram (Fig. 3A) resembles that of Fe- LPS. Both have lost peaks at retention times 362 and 425 seconds. The peak at 440 seconds seen in these samples appears as a very slight shoulder in the 425 second peak of non-electrodialysed Fe+ LPS (Fig. 2A, arrowed). The increment in calcium levels found in both Fe- and electrodialysed Fe+ LPS samples does not produce an HPLC pattern similar to that of Fe+ samples and this provides further evidence that calcium is unable to interact with *D. vulgaris* LPS to maintain its character as found in high iron environments. It also suggests that the stability-providing event occurs within the polysaccharide moiety of the LPS.

The quantity of monovalent potassium ions present in the LPS extracted from the two cell types is apparently similar. This is also true for sodium (Table 1). When the quantities of these ions are compared, it is seen that there is a larger amount of potassium present in LPS, indicating a preference of the LPS molecules for potassium. A small quantity of potassium is removed by electrodialysis, but it is replaced by sodium (Table 1). This suggests that potassium and sodium may bind to the same ionic sites in the LPS molecules. Potassium has an ionic radius of 1.33A while sodium has a radius of 0.98A. It may be that these are sufficiently similar to allow binding at the same locus. The function of these ions is not clear, but it is suggested that they form part of the cationic pack for the neutralisation of charges within the LPS molecule, hence helping to maintain its integrity.

RESUMO

Interações catiônicas em lipopolissacarídeo de *Desulfovibrio vulgaris*

Foram utilizadas as técnicas de cromatografia à liquido de alto desempenho (HPLC), análise por

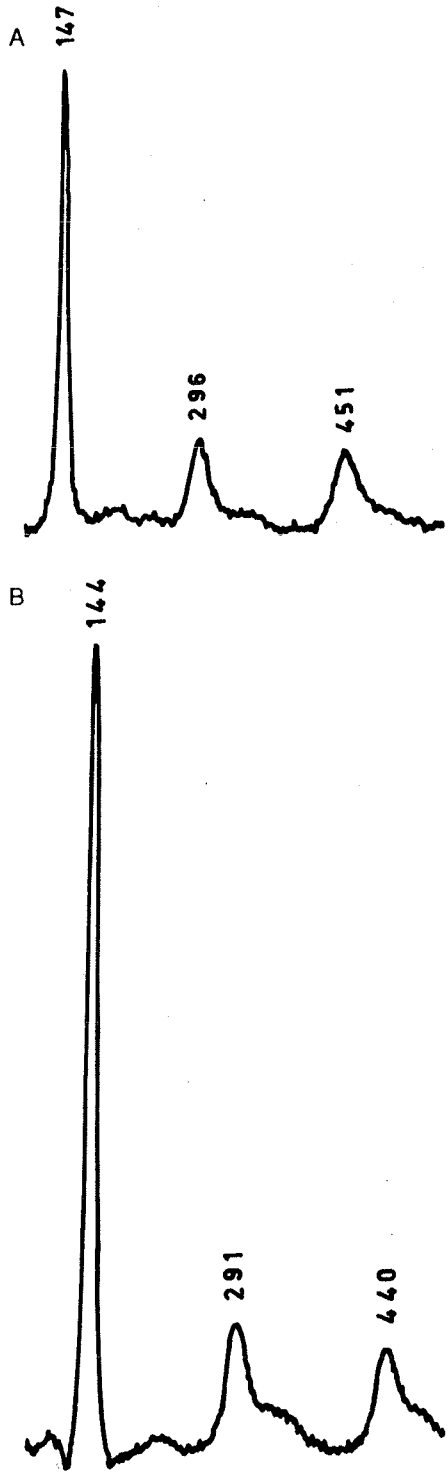

FIGURE 3 - HPLC sugar analysis of electrodialysed lipopolysaccharide extracted from *D. vulgaris* grown in iron-rich (A) or iron-poor (B) conditions. Numbers indicate retention times in seconds.

raio-X EDAX) e eletroforese em gel de poliacrilamida (SDS-PAGE) na investigação das interações entre vários cátions e o lipopolissacarídeo (LPS) extraído da espécie bacteriana *Desulfovibrio vulgaris*. O LPS demonstrou afinidades variáveis para diferentes íons divalentes. Eletrodiálise removeu a maioria dos íons Fe(II) do LPS e resultou num aumento dos íons Ca. A HPLC e SDS-PAGE demonstraram diferenças na estrutura de LPS isolado de células ricas ou pobres em FE(II), que indicaram que o Fe(II) pode atuar como um estabilizador de açúcares no interior da molécula. Esta função é específica para Fe(II), sendo que os níveis elevados de Ca em células pobres em Fe(II) não mantêm a estrutura do LPS. Os íons Na e K mostraram-se com capacidade semelhante para ligação ao LPS. Os resultados apresentam evidências adicionais na função sugerida do LPS de *D. vulgaris* na adsorção de Fe(II) do ambiente.

Palavras-chave: bactérias sulfato-redutoras; ligação de íons; *Desulfovibrio;* lipopolissacarídeo.

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BINDING OF CYPERMETHRIN RESIDUE IN BRAZILIAN SOILS AND ITS RELEASE BY MICROBIAL ACTIVITY

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SUMMARY

The presence of soil-bound cypermethrin residues was dependent on microbiological activity in a Humic Gley soil with a high organic matter content (8%), but not in a Red-Yellow Latosol where the clay component (75%) is greater than that of organic matter (1.3%). Bacteria isolated from Gley soil released part of the bound residue but lost such ability when cultivated in nutrient agar. Release of bound pesticide residue was detected again when the bacterial isolates where submitted to long-term culture in medium contaning mineral salts and cypermethrin as the sole source of carbon.

Key words: cypermethrin, soil-bound residues, soil microorganisms.

INTRODUCTION

Pyrethroids are widely used for their insecticidal activity. Ample information is available in the literature on their metabolism by animals, plants and soil yet little is know about their behaviour in Brazilian soils.

Recent results in our laboratory have shown that 60 days after the addition of ^{14}C-cypermethrin to a Humic Gley soil and a Red-Yellow Latosol soil, 28% of the pesticide was found soil-bound in the former and 48% in the latter. About 8 to 15% of this bound residue is available (3).

The aims of the present paper were to analyse some aspects of the role of microbiological activity on cypermethrin binding in Brazilian soils and to isolate soil microorganisms involved in the bioavailability of cypermethrin bound residues.

MATERIALS AND METHODS

Chemicals: Cypermethrin (-cyano-3-phenoxybenzyl (IRS) - cis-trans-3 (2, 2 dichloro vinyl dimethyl cyclo-propanecarboxylate)) uniformly labelled with ^{14}C in the benzyl ring (specific activity: 22,5μCi/mg), cis-trans isomer 3:7, 98% radiochemical purity, supplied by Shell Biosciences Laboratory (England) as well as the non-labelled cypermethrin (technical grade; 97,5% purity) were used.

Soils: A Humic Gley soil (pH 6.5; organic matter: 8,8%; sand: 20%; silt: 33%; clay: 47%) and a Red-Yellow Latosol (pH 5.1; organic matter: 1.3%; sand 10%; silt: 15%; clay: 75%) were used in this study. The Humic Gley soil was collected from a Banana field of the Instituto Biológico and the Red-Yellow Latosol from a non-cultivated area in the countryside of S. Paulo State. Soils were sampled from the 0-15 cm layer, air-dried and sieved through a 2mm sieve.

Soil treatment and Analysis:

Influence of Biological Activity - Triplicate samples of Humic Gley and Red-Yellow Latosol soils were placed into glass jars (250ml); the role of the microbiological activity on pesticide binding

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was verified by comparing the behaviour of sterilized soil samples with that of non-sterilized (control) samples. Sterilization was obtained by autoclaving triplicate samples at 120°C for one hour during 3 consecutive days. To bring the soil moisture content to 70% of its field capacity, sterilized distilled water or distilled water was added to the corresponding sterile and non-sterile soils. Flasks were kept in the dark at 30°C.

After one week, 1.0 ml of a radiolabelled plus unlabelled cypermethrin in acetone solution was added to the soils to give a pesticide concentration of 50μg/g and radioactivity of 0.6μCi/ml, followed by incubation at 30°C for 75 days. Distilled water was added as necessary to maintain the appropriate soil moisture content.

Samples were extracted with 50 ml of a hexane:acetone mixture (4:1) for 4h in a shaker. The radioactivity in the extracts was measured by counting 1.0 ml aliquots in a scintillation Counter (Beckman LS 5801), using a scintillation cocktail composed of 4.0 g of PPO (2,5-diphenyloxazole) and 250 mg of dimethyl POPOP 1,4 bis 2-(5-diphenyloxazole)-benzene in 1L of toluene: renex solution (6:4). To remove any extractable radiocarbon, the samples were then subjected to exhaustive Soxhlet extraction with 150ml of methanol for 24h. The non-extractable residues (bound residues) were then determined by combustion of triplicates of 100mg of the dried solvent-extracted soil in a Harvey Oxidizer, where the bound radiocarbon was oxidized to $^{14}CO_2$ and analyzed in a scintillation counter (3).

Production of Soil Bound Residues For Microbiological Studies - 0.5ml of a ^{14}C-cypermethrin acetone solution (total radioactivity counts: 640,000 dpm) was added to triplicates of 10g of Red-Yellow Latosol soil and the flasks incubated at 30°C for 120 days. Distilled water was added as necessary to maintain the initial soil moisture content. At the end of the incubation period, the samples were extracted as previously described and the cypermethrin soil-bound residue determined.

Isolation of Microorganisms - Ten grams of Humic Gley soil were suspended in 100ml of sterilized water. Fivefold serial dilutions of this soil suspension were used to inoculate triplicates of Erlenmeyer flasks (50ml) where 1.0g of autoclaved Red-Yellow Latosol soil containing ^{14}C-bound cypermethrin (2.8μg and 0.017μCi) had been suspended in 10ml of sterilized mineral salt medium ($NaNO_3$: 1.0 g/l; K_2HPO_4: 2.0 g/l; $MgSO_47H_2O$: O.5 g/l; pH 6.0). A small vial containning 0.5ml of KOH was attached to the plug of each Erlenmeyer to trap any $^{14}CO_2$ released from the bound cypermethrin residue by the microorganisms. Flasks devoid of inoculum were used as controls.

After 30 days of incubation at 28°C in the dark, the vials containing KOH were removed, the KOH mixed with 10ml of the liquid scintillation cocktail (PPO:4.0g:POPOP:200mg:Renex:370ml in toluene to 1L) and the radioactivity present as $^{14}CO_2$ counted in a liquid scintillation Counter (Packard Tri-Carb 1600).

Flasks with positive $^{14}CO_2$ release were used for the isolation of microorganisms: 0.1ml of the liquid suspension was streaked onto the surface of nutrient agar plates or of mineral salt agar medium enriched with 1.0ml of an aqueous solution of cypermethrin (100μg/50ml sterilized water), added to the melted agar after autoclaving. A mineral salt liquid medium enriched with cypermethrin (10mg/l) was also used to culture the microorganisms.

After growing for 7 days on agar plates and for 15 days in liquid medium, the colonies and suspensions of microorganisms were bioassayed again for their ability to release $^{14}CO_2$ from soil bound ^{14}C-cypermethrin residues.

RESULTS AND DISCUSSION

According to the present data, the biological activity of microorganisms is important for pesticide binding in a soil with higher organic matter content, as only 5.6% of the radioactive carbon remained bound in samples of this type of soil submitted to sterilization, in contrast with 41% bound pesticide found in the non-sterilized samples (Table1). However, when using the Red-Yellow Latosol, which is poor in organic matter, no differences were found between sterilized and non-sterilized samples (Table 1). The influence of soil organic matter on the

TABLE 1 - Influence of biological activity on the binding of ^{14}C-cypermethrin in soils. Percentage of radiocarbon extractable and bound after 75 days of incubation of ^{14}C-cypermethrin in soil.

Treatment	Humic Gley		Red-Yellow Latosol	
	Extractable	Bound	Extractable	Bound
Sterlized	94.0	5.6	38.0	57.0
Non-sterilized	59.0	41.0	42.0	57.0

formation of bound pesticide residues has been described and indicates that the pesticides or their metabolites are chemically bound to the soil's organic component (4, 6, 7). Some reports have also shown a high non-extractability of pyrethroids in soils poor in organic matter but rich in clay (5). The results described herein for ^{14}C-cypermethrin in Humic Gley and Red-Yellow Latosol suggest that in the latter case, besides the influence of the organic component, physical binding to the clay also plays an important role in the formation of soil bound cypermethrin residues.

Release of bound ^{14}C-residues as $^{14}CO_2$ evolution was detected with all serial dilutions of the Red-Yellow Latosol soil samples incubated for 30 days. The release corresponded to 11% for the twofold dilution and to 2.6% for the fivefold dilution. When the twofold dilution was plated onto nutrient agar, three bacterial colonies were isolated. However, these isolates were not very active with respect to the release of bound cypermethrin residues (Table 2). Subsequent transfer and growth of such isolates onto agar plates enriched with cypermethrin led to loss of their ability to use bound soil residues. Significative release of $^{14}CO_2$ from the soil bound ^{14}C-cypermethrin was re-established only when the bacteria were cultivated in liquid salt medium enriched with cypermethrin as the sole carbon source (Table 2).

TABLE 2 - Release of $^{14}CO_2$(%) from soil-bound 14C-cypermethrin after incubation with microorganisms isolated from Humic Gley soil.

Isolate nº	Nutrient agar	Cypermethrin agar	Liquid medium
#1	1.4	3.7	25.9
#2	3.5	3.5	22.7
#3	2.6	2.9	22.7

Although the microorganisms grew well on agar plates, the release of soil bound cypermethrin by pure cultures of such isolates could not be demonstrated; this suggests that the growth of bacteria in agar causes loss of their ability to use soil bound pesticides by some carry over of the nutrients during the plating procedure or to loss of indigineous plasmids (1, 2). The possibility of an effect derived from a mixed bacterial culture also has to be considered.

The bacteria responsible for the release of cypermethrin soil bound residues were not identified since, though positive results were obtained with the liquid medium culture, no significative release was detected with the isolated colonies grown in agar.

The yellow colonies of isolates #1 and #3 presented positive reaction as *Pseudomonas* in King B medium. According to the literature, the release of bound residues from soil treated with ^{14}C-atrazine was detected after incubation of the solvent extracted soil with two species of *Pseudomonas* (4).

The release of soil-bound cypermethrin residues by soil bacteria can be taken as evidence that these residues are not completely excluded from environmental interactions, although several aspects of the laboratory trials differ from field conditions, like constant temperature, optimal moisture content and use of an unformulated active ingredient.

RESUMO

Influência da atividade microbiana na liberação de resíduos de cipermetrina ligados ao solo

Resíduos de cipermetrina ligados ao solo são dependentes da atividade biológica em solo Gley Húmico, com alto teor de matéria orgânica (8%); não ocorre dependência em solo Latossolo Vermelho Amarelo onde o conteúdo em argila é alto (75%) e a matéria orgânica bem menor (1,3%). Isolaram-se bactérias de solo Gley Húmico que liberam parte desse resíduo ligado, porém, após cultivo em meio agar nutritivo, as bactérias perderam essa propriedade, recuperada após longo cultivo em meio de sais minerais com cipermetrina como única fonte de carbono.

Palavras-chave: cipermetrina, resíduos ligados ao solo, microrganismos do solo.

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ENTEROTOXIN OF *AEROMONAS* SP: SOME CULTURE CONDITIONS WHICH AFFECT ITS PRODUCTION

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SUMMARY

Strains of *Aeromonas sobria* and *Aeromonas hydrophila* isolated from several foods were checked for the production of enterotoxin (Infant Mouse Test) using different culture media (BHI, TSB and CAYE) and conditions of culture (variation of incubation temperature, of aeration and changes in the volume of medium / volume of flask ratio). The results showed no production of enterotoxin in CAYE medium. The presence of enterotoxin was detected at low temperatures (16° C) and higher volumes (60 ml) of medium and under stationary culture, suggesting that aeration and the temperature of incubation, though not critical, are important factors to be considered as far as *Aeromonas* enterotoxin production is concerned.

Key words: enterotoxin production, *Aeromonas* sp, culture conditions.

INTRODUCTION

Some studies (2, 3, 10 ,11) have demonstrated that *Aeromonas* sp exhibit several virulence factors such as production of enterotoxins and cytotoxins, adherence to intestinal cells and an invasive ability.

Presently, much attention has been given to an enterotoxin which is thermolabile yet is active in the infant mouse test as the thermostable STa enterotoxin from enterotoxigenic *E. coli* (12). This toxin, together with the other virulence factors, makes *Aeromonas* sp a potential enteropathogen for man (9, 12, 13).

The following species of *Aeromonas* are identified according to their motility and biochemical characteristics: five motile (*A. sobria*, *A. hydrophila*, *A. caviae*, *A. veronii* and *A. schubertii*) and two non-motile (*A. salmonicida* and *A. media*). Since raw foods, including vegetables and meats, can be a source of infection to man as far as diarrheal diseases are concerned, 15 strains of *A. hydrophila* and *A. sobria* isolated from foods were examined for the production of enterotoxin in this work. Futhermore, the influence of different culture conditions, such as temperature of incubation, use of shaking and changes in the volume of medium in relation to the flask's volume were also investigated.

MATERIALS AND METHODS

Strains

Nine strains of *Aeromonas sobria* and six of *Aeromonas hydrophila* were isolated from different

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TABLE 1 - Identification of the *Aeromonas* sp strains.

Strains	Species	Origin
36	*A. hydrophila*	chopped beef
38	*A. hydrophila*	chopped beef
40	*A. sobria*	chopped beef
50	*A. sobria*	fish meat
69	*A. hydrophila*	chopped lettuce
75	*A. sobria*	chopped beef
76	*A. sobria*	chopped beef
84	*A. sobria*	ground beef
91	*A. hydrophila*	cucumber, chicory
92	*A. hydrophila*	ground beef
93	*A. sobria*	ground beef
95	*A. hydrophila*	meat rolls
97	*A. sobria*	beef (fillet)
98	*A. sobria*	beef (fillet)
105	*A. sobria*	chopped beef

sources (Table 1). The bacteria were stored on trypticase soy agar until their examination for enterotoxin production as described next.

Production of enterotoxin

Volumes of 0.1 ml from a 9h-culture in Brain Heart Infusion were inoculated into 125 ml Erlenmeyers containing 10 ml of the following media: casaminoacids-yeast extract (CAYE) (8), trypticase soy broth (TSB) (Difco) supplemented with 0.6% of yeast extract and BHI broth (Difco). Flasks were incubated at 37° C under shaking (100 rpm) for 18 to 24h. Subsequently, the cultures were centrifuged at 3,000 rpm for 20 min and then at 10,000 rpm for 10 min. The supernatants obtained were stored at -25°C in Eppendorf tubes and examined for the presence of enterotoxin throughout the experimental work. The following culture conditions were also evaluated concerning their influence on enterotoxin production a) BHI medium (10 ml), incubation with shaking at 16°C for 18 - 24h; b) BHI medium (10ml), stationary incubation at 37°C for 18-24h; c) as in a) but with incubation at 37°C; d) as in e) but with incubation at 16°C.

Infant mouse test (IMT)

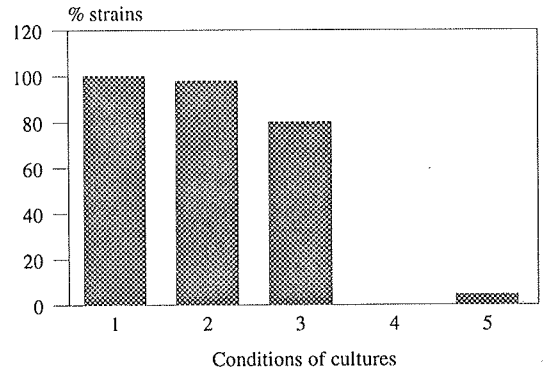
Swiss 3-5 days-old mice were used. Each bacterial strain was inoculated intragastrically into 3 animals using volumes of 0.1 ml per mouse. The animals were maintained at room temperature for 3h and then euthanized by ether inhalation and necropsied. The guts of every 3 animals inoculated with the same supernatant were taken out and the ratio of gut weight over the remaining carcass weight calculated. Results were recorded according to Serafim et al (14) as for the detection of thermostable enterotoxin (STa) from enterotoxigenic *E. coli* (ETEC). Values higher than 0.083 were considered positive. Conversely, lower values were considered negative (though seldom found, negative tests with values close to 0.083 were repeated and the new results always established whether a strain was definitely negative or positive). Whenever the IMT tests were performed to evaluate conditions which could influence enterotoxin production by *Aeromonas* sp strains, as listed in item 2, each IMT (3 mice per test) was repeated 4 times (n=4); the values obtained represent the mean of these data.

RESULTS

IMT tests carried out in BHI and TSB-YE media showed comparable results, all strains being positive for enterotoxin production. However, supernatants from such cultures did not show any enterotoxin activity when heated at 56°C for 10 min, as measured by the IMT test.

It is important to point out that enterotoxin production was not detected in CAYE medium.

Concerning the study of enterotoxin production under different culture conditions, Fig. 1 shows that incubation of 10 ml cultures at 37°C with shaking at 100 rpm did not influence secretion

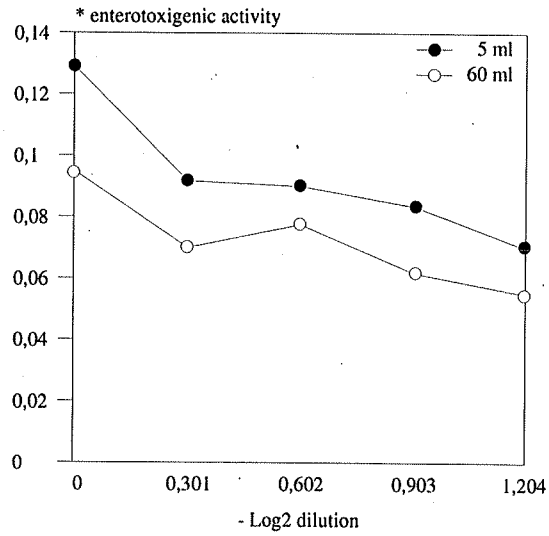


1: cultures incubated at 37°C with shaking (10 ml BHI)
2: stationary cultures incubated at 37°C (10 ml BHI)
3: cultures incubated at 16°C with shaking (10 ml BHI)
4: stationary cultures incubated at 16°C (10 ml BHI)
5: stationary cultures incubated at 16° (5 ml BHI)

FIGURE 1 - Frequency of the strains of *Aeromonas* sp producing enterotoxin as detected by the IMT test when cultures in BHI medium were incubated under different conditions. Strains tested are listed in Table 1.

of the toxin by the strains analysed. Incubation at 16°C, on the other hand, inhibited the production of enterotoxin in stationary cultures inside flasks containing 10 ml of BHI, yet 5% of the strains became positive by the IMT when testing these conditions using 5 ml volumes (Fig. 1)

To ascertain whether there could be any influence of the volume of media in relation to the volume of the flask, experiments were carried out and showed that incubation in medium volumes of up to 60 ml of stationary cultures of *A. hydrophila* (strain 69) at 37°C apparently did not affect the IMT results (data not shown). However, dilution of the secreted enterotoxin in the order to measure its potency as assayed by the IMT showed that the enterotoxigenic activity of 5 ml-cultures, whatever the dilution tested, was stronger than that observed for 60-ml cultures (Fig. 2). Four other strains, being 2 of *A. hydrophila* (n° 91 and 92) and 2 of *A. sobria* (n° 40 and 50), showed similar results but were not included in this figure to make it more understandable.



* Gut weight / carcass weight

FIGURE 2 - Enterotoxigenic activity (EA) under different dilutions of supernatants of stationary culture (strain 69 *A. hydrophila*) incubated in flasks containing 5 ml and 60 ml of BHI medium, at 37°C. The mean value of each EA data was obtained through 4 repetitions of each test (n = 4).

A similar experiment was carried out with strain 69 of *A. hydrophila* to evaluate the influence of the temperature of incubation (16°C and 37°C) on the secretion of enterotoxin. This experiment had to be done under shaking (100 rpm) since stationary cultures did not produce enterotoxin at 16°C. The results obtained for n=4 (number of repetitions of each test) did not show any significant difference between non-stationary cultures incubated at either 37°C or 16°C, suggesting that aeration might be more important than temperature of incubation within the range tested (data not shown). Similar results were also obtained with 2 strains of *A. hydrophila* (n° 91 and 92) and 2 strains of *A. sobria* (n° 40 and 50).

DISCUSSION

All *Aeromonas* strains were toxigenic by the IMT when grown in BHI and TSB-YE. Conversely, no toxin was produced by cultures inoculated in CAYE medium, which is routinely used for the detection of STa enterotoxin produced by ETEC. This observation suggests that this *Aeromonas* toxin might be different from the thermostable STa enterotoxin of ETEC, despite the fact that the biological responses in the IMT for both *Aeromonas* and ETEC toxins were identical. Because the IMT has been often used for the detection of thermostable STa enterotoxin of ETEC (14), there are some reports in the literature that refer as thermostable the *Aeromonas* sp enterotoxin detected by the IMT (4). However, it has been recently demonstrated that this enterotoxin is thermolabile (3) despite giving the "in vivo" response in baby mice which mimics that of the STa ETEC enterotoxin.

In fact, the IMT enterotoxin produced by *Aeromonas* is still a puzzling subject. Some authors (5,7) reported positive results only in the rabbit ileal assay and Burke et al (4) suggested that, besides the gut-carcass ratio, the observation of liquid diarrhea should be taken into consideration in the IMT assay, though this was not observed in our study. Thus, a positive IMT is enough to identify an enterotoxigenic *Aeromonas* sp.

The different experiments showed that aeration and an adequate temperature of incubation can be taken as important factors for the production of enterotoxin by *Aeromonas* sp. Our data have shown that aeration is likely to be as critical as it is for the secretion of STa and LT produced by ETEC (1,6). On the other hand, a decrease in temperature seemed to influence negatively the production of *Aeromonas* sp enterotoxin as detected by the IMT. The most appropriate temperature was 37°C, independently of a non-stationary incubation. At 16°C,

however, only cultures kept under shaking produced some *Aeromonas* enterotoxin, suggesting again that aeration has a more pronounced effect than temperature in the yield of this toxin; obviously, aeration (incubation under shaking) at 37°C gave a better production of *Aeromonas* sp enterotoxin, as shown in figure 1, columns 1, 2 and 3. It could be thought that temperature might be a more influential factor than aeration, yet column 5 of the same figure demonstrates that improved aeration (shaking) at 16°C yielded some enterotoxin production that was undetectable in column 4, which corresponds to cultures also incubated at 16°C but with reduced aeration (stationary cultures) (volumes of 10 ml of medium).

Figure 2 represents dilutions of *A. hydrophila* (strain 69) cultures incubated stationarily inside flasks containing different volumes of BHI; the results suggest that whatever the log2 dilution within the range of 5 sequential dilutions (from net to 10^{-1024}), enterotoxin production by this *A. hydrophila* strain was higher when aeration was more pronounced.

It is important to point out that the IMT is a semiquantitative test, at least with regards to the STa enterotoxin of ETEC, and that, although not often, higher mean values of gut/carcass weight ratios may be found with supernatants diluted at 1:4 than at 1:2 (Pestana de Castro, personal communication). Therefore, when looking at Figure 2, which shows reliable data, one must bear in mind that dose-response in the IMT test is not always correlated.

Experiments using shaking and different temperatures of incubation were carried out with 4 other strains of *Aeromonas*, including 2 *A. hydrophila* (nº 91 and 92) and 2 *A. sobria* (nº 40 and 50); additionally, each IMT value represents the mean (X) of 4 repetitions (data not shown). Based on the findings obtained, the following main conclusion can be drawn: type of medium, conditions of aeration and temperature of incubation are important factors to be considered for the production af *Aeromonas* sp enterotoxin as detected by the IMT test. Also based on our data, it is possible to say that some negative results reported in the literature for the *Aeromonas* IMT enterotoxin production are likely to be due to the use of CAYE medium, which is commonly very reliable for the production of ETEC STa enterotoxin (6) but was found to be inappropriate for the production of *Aeromonas* enterotoxin as detected by the IMT.

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RESUMO

Enterotoxina de *Aeromonas* sp: condições de cultivo que afetam sua produção

Cepas de *Aeromonas sobria* e *Aeromonas hydrophila*, isoladas de diferentes alimentos, foram submetidas ao teste de produção de enterotoxina (teste do camundongo recém nascido) usando diferentes meios de cultura (BHI, TSI e CAYE) e condições de cultivo (temperatura, aeração e volume de meio de cultura em relação à capacidade do frasco). Os resultados mostraram que não houve produção de toxina no meio CAYE. A presença de enterotoxina foi detectada em baixa temperatura (16°C) e em volumes mais elevados de meio (60 ml) e em culturas estacionárias, sugerindo que a aeração e a temperatura de incubação, embora não criticos, são fatores importantes na produção de enterotoxinas por *Aeromonas*.

Palavras-chave: Produção de enterotoxina, *Aeromonas* sp, condições de cultivo.

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STUDY OF MULTIRESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES FROM A BRAZILIAN HOSPITAL

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SUMMARY

Nosocomial infections with *Staphylococcus aureus* strains, resistant to multiple antibiotics have reached epidemic proportions in a teaching hospital in Ribeirão Preto, State of São Paulo, Brazil, over the last 5 years. Twenty-two strains of multiresistant *S. aureus*, isolated during 1988 from several clinical sources, were examined and revealed similar antibiotic sensitivities and other characteristics. The majority belonged to the phage group III, and were phage type 84; all possessed a single small plasmid of approximately 3.2 kb, which probably did not determine antibiotic resistance.

Key words: nosocomial infections; multiresistant *S. aureus, S. aureus* phage typing; *S. aureus* plasmids.

INTRODUCTION

Bacterial multiresistance to antimicrobial agents is an important nosocomial problem. One of the main factors in generating antimicrobial multiresistance is the seletive pressure brought about by indiscriminate use of broad spectrum antimicrobials and the consequent spread of resistant plasmids. Methicillin resistant *Staphylococcus aureus* (MRSA) appeared in Australia in 1960s, spread to Europe and the USA at the end of the 1970s, and are now disseminated word-wide(1,9).

Since 1984 MRSA have been isolated in a 600 bed teaching hospital, in Ribeirão Preto, São Paulo, Brazil. By 1987 nearly 70 patients had been colonized or infected by these bacteria(13). Many different approaches were attempted unsuccessfully to control or eradicate these strains from the hospital.

We selected 22 isolates among 120 MRSA, isolated during 1988, for study by phage typing, plasmid profiling and curing experiments.

MATERIALS AND METHODS

Bacterial cultures

The 22 isolates investigated were selected among 120 strains recovered from documented hospital infection in patients from several different

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hospital units; namely Intensive Care, Surgery, Burns, Infectious Diseases, Nursery and Orthopedics. These strains were isolated from wound infections (2 strains), urine (3 strains), blood (4 strains), sputum (1 strains), cerebrospinal fluid (2 strains), abscess (8 strains) and ocular (2 strains).

Strains were identified as *S. aureus* by the tube coagulase test, production of lecithinase, deoxiribonuclease (DNase) and thermostable nuclease (TNase), as previously described (7). All isolates were stored on LB medium (Tryptone 1%, yeast extract 0,5%, NaCl 1%, agar 1,7%).

Antibiotic sensitivity testing

Antibiotic sensitivity testing was carried out by the disc diffusion method of Bauer et al. (2). Müeller Hinton agar plates (MH, Merck Laboratories) and antibiotics discs (Cecon Laboratories, Brazil) containing the following amounts of antibiotics were used : chloramphenicol (C) (30 μg), erythromycin (E) (15 μg), gentamicin (G) (10 μg), lincomycin (L) (2 μg), tetracycline (T) 30 μg), penicillin (P) (10 UI), oxacilin (O) (1 μg) and vancomycin (V) (30 μg). MICs were performed according to recommended methods (14,20) using the two fold plate dilution method in MH agar with an inoculum of approximately 10^8 cfu ml^{-1}. The above antibiotics were diluted in water or phosphate buffer (21). *S. aureus* ATCC 25923 was used as control strain with previously recommended zone size criteria. MIC breakpoints were used to define all isolates in susceptible or resistant (14).

Phage Typing

Phage typing was performed by the standard method of Blair and Williams (4) by the National Phage Typing Reference Laboratory-Ribeirão Preto-SP-Brazil. The International set of phages were used, at RTD and 100x RTD.

Plasmid Analysis

Plasmid DNA was isolated by 3 methods : Dunkle & Sippel (8), Kado & Liu (11) and Birnboim & Doly (3), using lysostaphin (Sigma Chemical Co.) for staphylococcal plasmid extraction. The electrophoresis buffer was prepared with 890 mM Tris hidrochloride-basic oxide (pH 8,0) in 0,025 mM EDTA. Agarose type II, medium EEO (Sigma Chemical Co.) was dissolved to a final concentration of 0,8% in electrophoresis buffer. Cleared lysates (25μl) were mixed with 5 μl of a dye solution. All electrophoresis were performed on a horizontal apparatus, at 12 Vcm^{-1} for about 3 hs.

Plasmid elimination was achieved by growing *S. aureus* isolates in Brain Heart Infusion broth (BHI-Merck) at 42°C for 18-24 h (19) and also by growing isolates in BHI containing 3 to 4 μg/ml of ethidium bromide (Sigma Chemical Co.) at 37°C (5). When the broth became turbid, the strains were plated and single colonies from each culture were picked and screened to determine antibiograms on Müeller-Hinton agar containing the antibiotics according MIC determinations previously done.

RESULTS AND DISCUSSION

We have studied isolates from an outbreak of MRSA by using disc susceptibility, phage typing and plasmid analyses. The clinical source of the 22 MRSA isolates and their characteristics are shown in table 1. All 22 *S. aureus* isolates produced hemolysis, yellow pigment, lecithinase, plasma coagulase, DNase and TNase. The multiresistance of the 22 isolates studied was confirmed by MIC determinations. The resistance level of the isolates revealed an uniform and high resistance. Most isolates were resistant to penicillin, erythromycin, gentamicin, lincomycin, tetracycline and oxacilin; isolate 3 was susceptible to tetracycline and erythromycin and isolate 6 to lincomycin and chloramphenicol. As expected all isolates were vancomycin susceptible.

Phage typing revealed that isolates were predominantly of the group III and most of them reacted with phage 84, but all varied in phage typing pattern, i.e., 10 isolates reacted only with phage 84, 5 isolates showed different combination of these three phages, 54, 77 and 84, and 4 others reacted with other phages of group III.

There was one MRSA isolate of the phage group II, and this is unusual in nosocomial isolates (12).

Many of the strains at routine test dilution (RTD) were poorly reactive with the International Set of typing phages, but lytic reactions were obtained with phages at 100 RTD for all but two of them. 100 RTD was important, particulary to confirm those 2 NT isolates, and could also show other reactions for the four isolates that reacted with phage 84 on RTD. Many MRSA isolates

TABLE 1 - Characteristics of *S. aureus* isolates

Isolate	Material and Ward(a)	Date of isolation	Antibiotic susceptibility pattern(b) CGLPOETV	Phage Type(c)	Phage Group
1	abscess-SU	12 april 88	RRRRRRRS	84*	III
2	abscess-SU	20 april 88	RRRRRRRS	77/84*	III
3	wound-NU	26 april 88	RRRRRSSS	84*	III
4	abscess-ORT	2 may 88	RRRRRRRS	54/84*	III
5	abscess-SU	2 may 88	RRRRRRRS	84*	III
6	abscess-NU	28 april 88	SRSRRRRS	54/77/84*	III
7	urine-SU	22 march 88	RRRRRRRS	6/42E/47/54/75/77/84	III
8	abscess-SU	28 march 88	RRRRRRRS	84*	III
9	ocular-OFT	16 march 88	RRRRRRRS	84*	III
10	abscess-ORT	31 march 88	RRRRRRRS	84	III
11	abscess-SU	22 march 88	RRRRRRRS	77/84*	III
12	blood-SU	7 april 88	RRRRRRRS	84	III
13	blood-BU	28 march 88	RRRRRRRS	84	III
14	wound-SU	10 may 88	RRRRRRRS	NT*	-
15	ocular-NU	16 may 88	RRRRRRRS	42E*	III
16	CSF-IDU	23 may 88	RRRRRRRS	NT*	-
17	blood-SU	18 may 88	RRRRRRRS	3A	II
18	urine-IDU	24 may 88	RRRRRRRS	83A/84*	III
19	urine-IDU	25 may 88	RRRRRRRS	42E/47/53/54/75/77*	III
20	blood-SU	26 may 88	RRRRRRRS	84*	III
21	CSF-IDU	6 june 88	RRRRRRRS	54/84*	III
22	sputum-IDU	31 july 88	RRRRRRRS	84	III

(a) SU- surgery unit, NU- nursery unit, ORT- orthopedics unit, BU- burns unit, IDU- infectious diseases unit, OFT- oftalmology unit, CSF- cerebrospinal fluid.
(b) C- chloramphenicol, G- gentamicin, L- lincomycin, P-penicillin, O-oxacilin, E- erythromycin, T- tetracycline, V- vancomycin, R-resistant, S- sensible.
(c) NT- nontypable; * indicates 100 RTD.

from different countries are weakly sensitive or are not typable with the current International Set of phages (1, 9, 6) and there is a need for the introduction of new typing phages (16,17) as well as for additional typing methods (22).

Since plasmid preparations for staphylococci may have some technical problems three different methods were used. Plasmid analyses showed one plasmid of approximately 3.2 kb in all 22 isolates. This plasmid belongs to class I of the staphylococcal plasmids, which could encode an unique resistance gene or could be a cryptic plasmid (15). We were unable to eliminate this plasmid from any of the isolates by our curing experiments. The cryptic plasmid rarely is cured "in vivo" or "in vitro"(19).

Usually the MRSA strains have one large plasmid and sometimes associated with a small cryptic plasmid (10).

The lack of large plasmids or the presence of small plasmids as found in all our isolates is not common and did not allow us to use the plasmid profiling as a typing tool for these MRSA isolates.

In Brazil, studies that were made after this confirm our findings. *S. aureus* oxacilin sensitive or resistant, from many Brazilian hospitals showed identical plasmid profiles : lack of large plasmids and presence of only two small ones (or 2 different conformations of the same plasmid), with less than 6 kb (18).

We conclude that the MRSA outbreak in our Hospital mainly involved isolates of phage group III with a small plasmid, propably not related with the multiresistance. Chromossomal genes or some transposons might be involved in these multiresistance.

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RESUMO

Estudo de *Staphylococcus aureus* multirresistentes isolados em um hospital brasileiro

Infecção hospitalar por *Staphylococcus aureus* multirresistentes atingiu proporções epidêmicas no Hospital das Clínicas de Ribeirão Preto-USP, nos últimos 5 anos. Vinte e duas amostras isoladas em 1988 de várias enfermarias foram examinadas e revelaram características semelhantes quanto à sensibilidade a antibióticos. Também, a maioria das amostras pertencia ao fagogrupo III, fagogrupo 84 e todas possuiam um único plasmídio de baixo peso molecular, com aproximadamente 3,2 kb, que, provavelmente, não codifica genes de resistência a antibióticos.

Palavras-chave: infecção hospitalar, *S. aureus* multirresistentes, fagotipagem de *S. aureus*, plasmídios de *S. aureus*.

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BOVINE ROTAVIRUS IN THE STATE OF GOIAS

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SUMMARY

This study was conducted to provide data on rotavirus infection in cattle of the State of Goias, Brazil. 223 samples of diarrheal faeces of calves from dairy herds were collected from different districts of the state. When the Polyacrylamide Gel Electrophoresis (PAGE) test was applied, 16 (7.17%) of the collected samples showed the presence of the rotavirus. Of these, only 11 could be analysed concerning viral RNA and the results indicated the existence of three electropherotypes: "A" (one sample), "B" (two samples) and "C"(eight samples) The differences appeared in segment 1 for type "A" and in segments 7, 8 and 9 for types "B"and "C". According to the results of the Immunoenzymatic Assay (EIA), all the samples belong to group A, of the "typical" rotavirus.

Key words: rotavirus, bovine, electrophoresis, immunoenzymatic assay.

INTRODUCTION

Rotaviruses were first reported by Moebus et al. in 1969 (18) and have been described as important pathogenic agents of the enteric syndrome in mammals (domestic and wild), fowls and even in children (4,8,9,16,24,25,27,33) in many parts of the world, occurring particularly among young animals.

In bovines, rotaviral infection has been reported by many authors that describe differences in frequencies of about 10 to 80% (3,6,7,14,19,20).

In Brazil, the infection was first described by Linhares et al in 1984 (15), who reproduced the disease experimentally in gnotobiotic calves. Subsequently, the presence of rotaviruses was described by many authors not only in calves (7,11,12), but also in other species (1,5,16,26,27) demonstrating the growing interest on this infection in our country.

Rotaviruses have been identified by searching the viruses directly in the stool of animals, diarrheic or not, through techniques like Electron Microscopy (EM), Immunoenzymatic Assays (IEA), Polyacrylamide Gel Electrophoresis (PAGE), Counterimmunoelectroosmophoresis (CIEOP), Cell Culture (CC) with many kinds of cell lineages, and others. Of these methods, the most commonly used are the PAGE test, which selects viruses in all groups by analysing the electrophoretic features of the RNA, and the IEA, which was only used to detect the group A viruses and is based on the detection of the inner capsid's VP6 antigen. Both methods are quite sensitive for detection of infected animals (3,4,23).

In an attempt to classify human rotaviruses, Lourenço et al (17) suggested the term "electropherotype" to denote differences between some isolates without establishing antigenic differences among them. According to Bridger, 1987, (2), using this technique it is possible to distinguish distinct groups easily, since each group normally exhibits a specific feature.

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Roger & Holmes, 1979, (28) reported the presence of eight electropherotypes in bovine faecal samples. Four electropherotypes of Rotavirus were detected by Kalica et al, 1978, (13) and four by Jerez et al, 1989, (12) in diarrheic faecal samples from calves.

The aim of the present investigation was to obtain data on bovine rotaviral infection in Goias, Brazil, reporting the presence of the pathologic agent, showing its frequency and, by means of the PAGE and IEA techniques, determining the group to which the isolates belonged as well as the electopherotypes of the identified viruses.

MATERIALS AD METHODS

Faecal Samples (field samples)

223 samples of faeces from diarrheic calves from Holstein, Gir and Girolanda herds were collected. The samples were taken from calves aged between 1 and 120 days belonging to 34 dairy herds from 14 districts of Goias (Figure 1). Of these herds, 5 had to be sampled twice: 4 after presenting a positive result for rotavirus infection folowing the first sample collection and 1 to solve

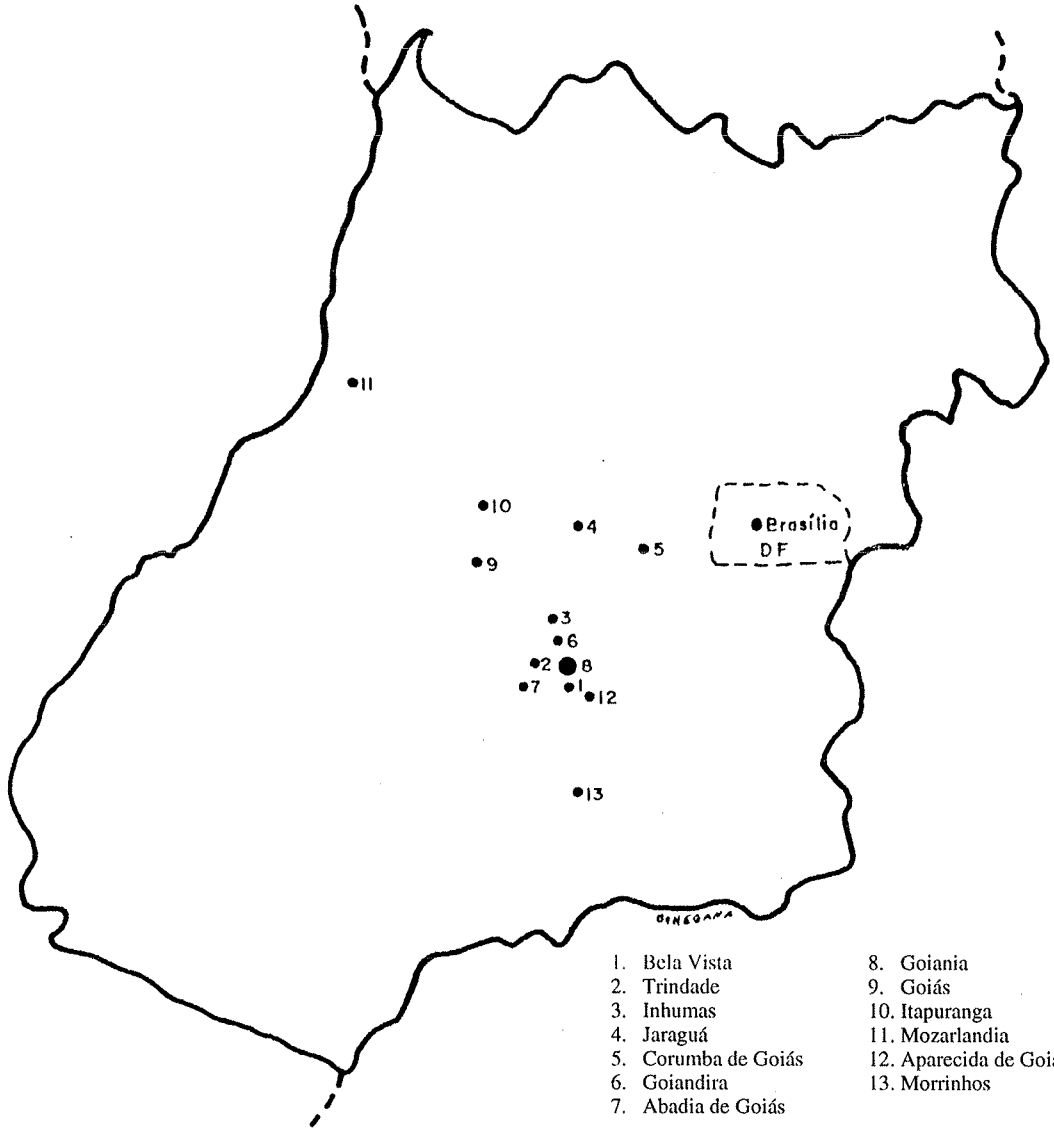

FIGURE 1 - Districts where samples were collected for the rotavirus survey on calves from the State of Goiás, Brazil.

TABLE 1 - Sample distribution for each district/property.

Districts	Property	Samples / Property	Positives
Abadia de Goiás	A*	18	-
Aparecida de Goiania	B1	1	1
	B2	1	-
	B3	3	-
	B4	3	-
	B5	2	-
	B6	1	-
	B7	4	2
Bela Vista de Goiás	C1	6	1
	C2	8	-
	C3	3	1
	C4*	12	2
	C5	4	-
Corumbá de Goiás	D	4	-
Goianésia	E	4	-
Goiânia	F1	6	1
	F2	12	-
	F3*	20	1
	F4	12	-
Goiás	G	11	-
Inhumas	H1*#	11	2
	H2	10	-
	H3	7	1
	H4	5	1
	H5	3	-
Itapuranga	I	5	-
Jaraguá	J	5	-
Morrinhos	K	4	-
Mozarlândia	L1*	7	-
	L2	16	2
	L3	5	-
Trindade	M1	4	-
	M2	3	1
	M3	4	-

Legend: (*) = Properties which were visited twice
(#) = Property where rotavirus was found once again.

a problematic situation.The distribution of samples per herd and per district is presented in Table 1.

Samples were obtained by carefully introducing a Kahm tube in the rectum of each animal. The tubes were then replaced by sterile plastic flasks with 50 ml capacity for better packing and transportation. Each flask was labelled with the name or number of the corresponding animal, its age and origin (owner and district) and transported on ice to the laboratory, where samples were diluted 10 to 20% in PBS, clarified for 15 minutes at 4°C (Sorvall RC 3C; 2600g) and then stored at -20°C.

Control Sample

The SA11 virus, cultured at the "Laboratório de Virologia"- ICB/USP was used as standard for positive controls.

Virological Analysis

The PAGE technique was employed to detect viral RNA in faeces according to Pereira et al, 1983, (23). Positive samples were confirmed by IEA (22) using the EIARA diagnostic kit for rotaviruses and adenoviruses (22) and the data were also used for classification.

Following the PAGE methodology, a total of 500 µl of each sample was placed in eppendorf tubes and 10% SDS added to reach a final concentration of 1%; the tubes were then kept at 37°C for 30 minutes. Next, RNA was extracted using a mixture of phenol/chloroform (v/v). After centrifugation, 50 µl of 20% NaCl and 500 µl of ethanol were added to the separated supernatant, which was left for 18 hours at a low temperature (-20°C) to permit RNA precipitation. The supernatant was then decanted and the precipitate treated with 25 µl of a dissociating solution (0.0625M Tris/HCl pH 6.8, 5M urea, 5% 2-Mercaptoethanol, 3% SDS and 0.01% Bromophenol blue) at 56°C for 15 minutes.

The electrophoresis was performed on polyacrylamide gel in a vertical cube using Tris/Glycine buffer (pH 8.3) and applying a 40 mA tension for 2 to 3 hours. Finally, the upper gel was removed and the lower gel stained by the silver nitrate method (10).

Positive samples were also analysed by the IEA "sandwich method" using the EIARA diagnostic kit made by the "Departamento de Virologia"/Fundação Oswaldo Cruz, Rio de Janeiro, Brazil; the kit was used for detection of both rotavirus and adenovirus.

Data Analysis

Samples were considered positive if, when submitted to the PAGE method, showed eleven well defined segments. They were then stored for retesting and later classified according to the

electropherotype (17). The IEA test was used to confirm a sample's classification into group A.

RESULTS AND DISCUSSION

The results are described in Tables 1 and 2. According to the PAGE data obtained, 16 (7.17%) of the 223 samples exhibited the presence of the eleven rotavirus segments, proving that the virus does occur among dairy cattle in the state of Goias.

TABLE 2 - Eletrophoretic patterns found in the positive samples detected by PAGE according to Lourenço et al, 1981.

Samples n#	Age (days)	Electropherotypes	Electrophoretic patterns			
			I	II	III	IV
016	60	1	b	a	g	b
034	30	?	*	*	*	*
036	?	?	*	*	*	*
041	30	?	*	*	*	*
065	15	1	b	a	g	b
071	20	?	*	*	*	*
236	30	1	b	a	g	b
237	30	1	b	a	g	b
283	08	1	b	a	g	b
294	30	1	b	a	g	b
295	45	?	*	*	*	*
298	30	1	b	a	*	b
303	25	1	b	a	g	b
351	?	1	b	a	g	b
372	15	1	b	a	g	b
373	12	1	#	s	g	b

Legend: (*) = indefinite motility
(#) = different segment motility when compared to published data from the bibliography

Similar data were found in the state of Minas Gerais by Fernandez et al, 1986, (7). Comparing these data with the ones described by other authors in Brazil and in other countries (3,6,11,14,19,20), we may consider that the infection rate in the analysed samples is relatively low. Tzipori, 1981, (32) comments that the infection rate for rotaviruses can vary according to different factors, such as environmental, immunological and even genetic factors. Management can also influence the infection rate.

Positive samples were stored and tested again at the end of the work to obtain a global result, aiming at analysing different electrophoretic profiles among the isolates. On retesting, only 11 of the 16 initially positive samples showed a well defined presence of the eleven rotavirus bands, while the remaining 5 did not allow an analysis of the segments.

The occurrence of initially positive samples changing into negative by SDS PAGE was reported by San Juan et al, 1986 (30) as probably caused by RNA degradation, which is likely to take place after freezing and thawing during laboratory procedures.

All the isolated samples submitted to IEA proved to belong to group A; according to the electrophoretic motility of segments 10 and 11, they probably belong to sub-group I - short pattern, as reported by Lourenço et al, 1981 (17) (Figure 2). The IEA gave no positive results for adenoviruses.

The highest frequency of group A was confirmed, which contributed to explain why rotaviruses of this group are considered "typical" (2).

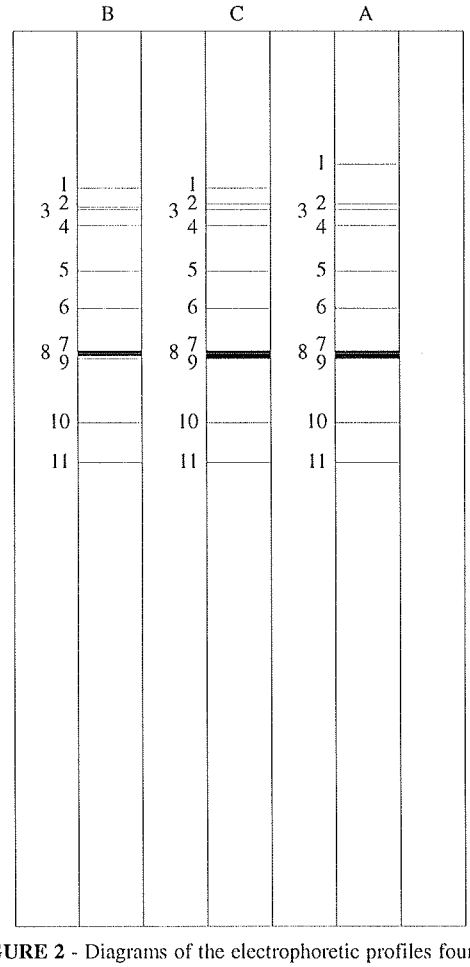

FIGURE 2 - Diagrams of the electrophoretic profiles found in calf faeces by PAGE.

The electropherotypes seen in the positive samples were grouped into three different patterns called A, B and C in this paper. The differences observed are shown in Figure 2 and were found at the class I level for electropherotype A (mobility of the first segment) and at the class III level for electropherotypes B and C (mobility of segments 7, 8, and 9).

The presence of more than one electropherotype has also been reported by other authors (12,13,21,29,30), the greatest differences described occurring mainly at the class III level where segments 7, 8 and 9 were present and in segment 1 class I, similarly to what was found in the present wok. The authors also reported differences in class II segments that were not detected in this work.

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RESUMO

Rotavirus bovino no Estado de Goiás

Esse estudo foi conduzido para obtenção de dados sobre infecção por rotavirus no gado bovino do Estado de Goiás, Brasil. 223 amostras de fezes diarreicas de bezerros de gado leiteiro foram coletadas em diferentes regiões do Estado. Quando a Eletroforese em Gel de Poliacrilamida foi utilizada, 16 (7,17%) das amostras coletadas acusaram a presença de rotavirus. Entre essas, apenas 11 puderam ser analisadas quanto ao RNA viral e os resultados indicaram a existência de três tipos eletroforéticos: "A" (uma amostra), "B" (duas amostras) e "C" (oito amostras). As diferenças ocorreram no segmento 1 do tipo "A" e nos segmentos 7, 8 e 9 dos tipos "B" e "C". De acordo com os resultados do Ensaio Imunoenzimático (EIA) todas as amostras pertenceram ao grupo "A", dos rotavirus "típicos".

Palavras-chave: rotavirus, bovino, eletroforese, ensaio imunoenzimático.

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TOXINS OF *MORAXELLA BOVIS* : EFFECT ON SUBSTRATES AND CELLS

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SUMMARY

Whole cultures and filtrates of twelve strains of *Moraxella bovis* were tested for the presence of gelatinase, DNAse, lecithinase and dermonecrotoxins and for their cytotoxic effect on five cell lines. Gelatinase and DNAse were detected in whole cultures, while dermonecrotoxins and cytotoxins for BHK 21 (Cl. 13) monolayers were demonstrated in the filtrates. The activity of the toxins present in whole cultures and filtrates was lost after heating at 100°C. The implications of these findings in the pathogenesis and immunological control of infectious bovine keratoconjunctivitis are discussed.

Key words: *Moraxella bovis*, exotoxins, cytotoxicity.

INTRODUCTION

The pathogenicity of *Moraxella bovis*, the etiologic agent of Infectious Bovine Keratoconjunctivitis (IBK) (5), is due to adhesins that act as primary factors (1, 6, 13, 18) and to toxins that affect several substrates present in the corneal and conjunctival cells, their target tissues. It has been shown that after adhering to the outer layers of the cornea, *M. bovis* invades the stroma, modifying the regular disposition of the fibrils of collagen, necrotizing the cells and producing an ulcer (7, 19).

Several toxins of this bacteria have been detected and studied, being also proposed as candidates for vaccines against IBK. Ostle and Rosembusch (17) studied an hemolysin produced by pathogenic strains of *M. bovis* and postulated its use as vaccine. They found that this toxin is associated to the surface of the bacterial cell, and that it is gradually destroyed after the end of the exponential phase of growth. Henson and Grumbles (15) reported that some strains produced dermonecrotoxins, while Gerber et al (11) used as vaccine pili and a protease that digested milk protein.

The aim of the present work was to study the effect of cell associated and of secreted toxins of *M. bovis* on several substrates and on established cell lines.

MATERIALS AND METHODS

Isolates

Twelve isolates of *Moraxella bovis* recovered from diseased animals as previously reported (12) and characterized biochemically (10) were studied. Their identification, origin and the passage level at which they were used are indicated in Table 1.

Toxin production

The isolates were grown in Tryptic Soy Broth (TSB)(Difco Laboratories, Detroit, Michigan USA) at 30°C for up to 40 hours in stationary

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culture. Half of each culture was filtered through a 0.22 mμ pore membrane. Whole cultures, filtrates, whole cultures heated at 100°C for one hour and filtrates heated at 100°C for 15 min. were tested for toxins.

TABLE 1 - Identification and origin of the strains of *Moraxella bovis* studied.

N°	Identification	Passages	Origin
1	JUR 3 A	7-8-9-10	Rio Grande, RS, Brazil
2	JUR 2 BO	9-11	Rio Grande, RS, Brazil
3	U 10	13-15	Uruguay
4	JUR	12-14-15	Rio Grande, RS, Brazil
5	Bagé	7	Bagé, RS, Brazil
6	U 3	14-16-35	Uruguay
7	Ar 2	19-22	Santa Vitória do Palmar, RS, Brasil
8	U 13	8	Uruguay
9	U 12	12	Uruguay
10	2358/08	17	Uruguay
11	SP 1	7	Uruguay
12	V 3	6	Dom Pedrito, RS, Brazil

Whole cultures and filtrates were tested for gelatinase production by the method of Fraser (10) and for DNAse using DNAse agar (4). Wells of 0.3 cm in diameter were done in the agar, filled with whole cultures or filtrates and the plates incubated at 37°C for 48 hs. Gelatinase and DNAse positive cultures were also inoculated as controls. Lecithinase was detected using egg yolk lecitho-vitelin as substrate (9). Briefly, 500 μL of whole culture or filtrate were added to 4,5 mL of calcium acetate solution (0.8 g of calcium acetate and 8.5 g of sodium chloride in 1000 μL of distilled water). An equal volume of egg yolk emulsion (one egg yolk in 250 mL of distilled water) was then added, thoroughly mixed and incubated at 37°C for 24 hs. Negative and positive controls were also used. Dermonecrotoxins were assayed by intradermal inoculation of 0.2 mL of whole cultures or filtrates on opposite sides of the same guinea pig. The reactions were observed and recorded daily during one week.

Effect on cell cultures

Whole cultures, filtrates and heated filtrates were placed on each of the following cell cultures grown to confluency on multiwell plates (Costar, Mass., USA): BHK 21 (Cl. 13) (ATCC CCL 10), CRFK (ATCC CCL 94), MDCK (ATCC CCL 34) and IBRS-2 (Centro Panamericano de Febre Aftosa, Rio de Janeiro, Brazil). One hundred μL of each suspension were added to an equal volume of Minimum Essential Medium (Gibco Labs., NY, USA) containing 10% bovine fetal serum and 100 μg of streptomycin and placed on the monolayers. The plates were kept at 25°C and observed daily with an inverted microscope for one week. Untreated cell cultures were kept as controls.

RESULTS

Toxins

Gelatinase was produced by 11 of the 12 isolates tested. Strain JUR (at passages 12 and 14) was the only one that did not produce the toxin. This toxin was detected in whole cultures but not in whole cultures heated at 100°C nor in the filtrates. DNAse was detected in whole cultures of all the isolates tested, but not in the filtrates. Lecithinase was not produced by the isolates tested.

Dermonecrotic lesions produced by the filtrates of all the isolates studied were characterized by hyperemia and edema 24 hours after inoculation, evolving to ulcers with areas varying from 12 to 60 mm^2 (average 31 mm^2) in 48 hours. Whole cultures produced a transient hyperemia that disappeared 48 hours after inoculation. Dermonecrosis was not induced by filtrates previously heated at 100°C for 15 min.

Cytotoxic effect

A cytotoxic effect was produced in BHK 21 (Cl. 13) cells by unheated filtrates of all the isolates studied, but not by whole cultures and filtrates heated at 100°C for 15 min. The effect was first seen 24 hours after the addition of the filtrate, the monolayer being completely destroyed at 48 hs while the controls remained unchanged. The other cell lines were not affected.

DISCUSSION

The characterization of toxins produced by bacteria is used for their classification by classical microbiological methods. Toxin studies, however, are also important to understand the pathogenic mechanisms in which they are involved.

Fraser and Gilmour (10) found that six of the seven *M. bovis* isolates they studied produced gelatinase, an enzyme related to the pathogenicity of several bacterial species. We detected this enzyme in whole cultures but not in the filtrates or

in heated cultures, suggesting that the toxin is associated with actively growing bacterial cells, likewise the hemolysin produced by this bacterium (15, 17). DNAse was also detected only in whole cultures, showing that it is not secreted to the culture medium in detectable amounts . The fact that these two toxins are found only in whole cultures but not in the filtrates could explain the observations that, after adhering to the superficial layers of the cornea, *M. bovis* invades the stroma disrupting the collagen fibers (7). Invasiveness is proper of bacteria that produce weak toxins or toxins that do not diffuse easily. This may suggest that toxins released by *M. bovis* are not essential antigens to be used in vaccines, making difficult to understand the conclusion of Gerber et al (11) that a milk digesting protease should be incorporated to vaccines. Probably, the results they obtained with the vaccine were more directly related with pili antigens than with the presence of the enzyme.

Lecithinase, the third enzyme investigated, was not detected among the isolates used, suggesting that the hemolysin produced by them is not a phospholipase C (20). Clinkenbeard & Thiessen (8) showed that this toxin causes pore forming cytolysis.

The isolates we studied produced dermonecrotoxins, confirming the findings of Henson & Grumbles (15) thirty years ago. These authors, however, considered that the toxins were associated to the cell wall, whereas in our experiments they were detected in filtrates and not in whole cultures. In another study, we found that several isolates recovered from diseased animals also produced dermonecrotoxins, irrespective of their plasmid profile (Gil Turnes, Aleixo, Dellagostin & Ribas, Proc. XIV Cong. Bras. Microbiol., Ribeirão Preto, 1989). The dermonecrotoxins are heat labile and produce their action independently of the presence of endotoxins. On the other hand, the reactions presently observed after the inoculation of whole cultures were similar to those described by Araújo et al (2) with the lipopolisaccharide of this bacterium. The possible role of these toxins in the pathogenicity of *M. bovis*, as happens with *Bordetella bronchiseptica* (16) and some Gram positive bacteria (3), is worth studying.

All the filtrates that produced dermonecrosis were cytopathic for BHK 21 (Cl. 13) cells, but not for the other cell lines used. Whole cultures and heated filtrates lacked cytotoxic and dermonecrotic activities, showing that the toxins responsible for these actions are thermolabile and that cell wall components are not involved. Annuar and Wilcox (1) found that five *M. bovis* strains adhered to MDBK and Bovine Corneal Epithelium cell lines, but not to BHK (Cl. 13) cells, suggesting that only cells of bovine origin have receptors for the adhesins of this bacterium. Our findings suggest that BHK (Cl. 13), a cell line affected by several bovine pathogens, also has receptors for the toxins of *M. bovis*, and that, even lacking those for their adhesins, make it a good cell target for the study of toxin action.

It is possible that other toxins are produced by pathogenic strains of *M. bovis* and that they may participate in the events that produce the disease. It has been shown, however, that avoiding the first step of the pathogenic process, inducing immunity against adhesins, the disease may be controlled (14).

RESUMO

Toxinas de *Moxarella bovis*: efeito em substratos e células

Culturas totais em meio líquido e filtrados, de 12 cepas de *Moraxella bovis,* foram testados para determinar a presença de gelatinase, DNAse, lecitinase e dermonecrotoxinas. Foi também estudado seu efeito citotóxico sobre cinco culturas celulares de linhagem. Nas culturas totais demonstrou-se a presença de gelatinase e DNAse. Os filtrados produziram dermonecrose em cobais brancas e efeito citotóxico em células BHK 21 (Cl. 13), mas não nas outras linhagens. A atividade tanto das culturas totais quanto dos filtrados foi eliminada por aquecimento a 100°C. Discute-se a participação destas toxinas na patogênese e no controle da Ceratoconjuntivite Infecciosa Bovina.

Palavras-chave: *Moraxella bovis,* exotoxinas, citotoxinas

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OCCURRENCE OF *SALMONELLA* SP AND *CAMPYLOBACTER* SP IN CHICKENS DURING INDUSTRIAL PROCESSING

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SUMMARY

The occurrence of *Salmonella* sp and *Campylobacter* sp in chickens examined after slaughter and during chilling of packaged carcasses in an industrial processing plant of Santa Catarina (Brazil) was studied. *Campylobacter* sp and *Salmonella* sp were detected in 56.6% and 10.0% of chicken cecal samples examinated just after slaughter, respectively. After processing, surface analysis for *Campylobacter* sp and rinsing for *Salmonella* sp revealed contamination levels of 50.0% and 13.3%, respectively. Additionally, a greater incidence of *C. coli* than of *C. jejuni* was detected in both cecal and surface samples; only one *Salmonella* serotype - *S. heidelberg* - was isolated.

Key words: *Salmonella* sp; *Campylobacter* sp; chickens; industrial processing.

INTRODUCTION

Poultry has been frequently held responsible for the transmission of foodborne disease to man, including campylobacteriosis and salmonellosis (5,15,44).

According to De Boer & Hahné (10), undercooking and cross-contamination of cooked foods with raw chicken are the main causes of *Campylobacter* infections associated with chicken products. These researchers reported that *Campylobacter* sp and *Salmonella* sp are responsible for about 15 and 5%, respectively, of all cases of human acute enteritis in the Netherlands.

Numerous studies have shown that some pathogens are frequently found in the gastrointestinal tract of poultry arriving to the slaughterhouse. The occurrence may vary from 25 to 100% and other sources of pathogens can contribute to carcass contamination during normal salughtering and processing operations, as *Salmonella* sp and *Campylobacter* sp (18,32,42).

Despite the level of chicken carcass contamination with *Salmonella* sp being normally low at the beginning of processing, dissemination of the microorganisms frequently occurs in the scalding tank by mechanical action and during the defeathering and evisceration steps and, furthermore, the permanence or even increase of those enteropathogens at the final processing step is predictable (27,50).

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In attention to recommendations of the US Advisory Committee on *Salmonella*, FSIS (USDA) reported that the recorded national incidence rate of *Salmonella*-contaminated broiler chickens in surveys conducted between 1982 and 1984 (35.2%) was virtually the same that the rate recorded in 1979 (36.9%) for the same chicken eviscerating plants (14,48). Regarding serotypes isolated during both surveys, *S. heidelberg* and *S. typhimurium* were the most frequently found species (13,14,48).

Examining 182 chicken carcass samples acquired from butcheries in Campinas (SP), Leitão (23) found that 4.9% of them were contaminated with *Salmonella* species.

Bokanyi Jr. et al. (7), searching *Salmonella* sp in ready-to-cook broiler or parts, showed that 43% of the samples were positive for *Salmonella* sp, the most frequently isolated serotypes being, by far, *S. hadar, S. heidelberg,* and *S. johannesburg,* which constituted almost half of the total cultures analyzed.

Several studies have shown that *C. jejuni* is a natural inhabitant of poultry and other domestic and wild animal faeces. A search carrried out in The Netherlands revealed the presence of *C. jejuni* in 61% of intestinal contents sampled from pigs (36); in the UK, between 14 to 91% of the intestinal tract of chickens showed the presence of *Campylobacter* jcjuni (45,52,53).

According to Doyle (11), 30 to 100% of poultry, 40 to 60% of bovine and 60 to 80% of swine carry *C. jejuni/coli* in the intestinal tract.

In Brazil, at least two studies have shown the presence of *C. jejuni/coli* in the intestinal tract of poultries (24,26).

Searching *C. jejuni/coli* in a highly automatized poultry processing plant in Norway, Rosef et al. (47) demonstrated the presence of these microorganisms in 31.3% of chicken surface samples examinated just prior to packaging.

Kwiatek et al. (22), after examining 839 poultry carcasses sampled immediately after defeathering and eviscerating but before chilling in a processing plant in Poland, showed that 80.3% of the chickens, 48.0% of the ducks, and 3.0% of the turkeys were contaminated with *Campylobacter* sp. Similar results were found by De Boer & Hahné (10) in The Netherlands, and *C. jejuni* was isolated in 61.0% of 279 samples of chicken taken from retail outlets.

Studies conducted in Brazil have indicated the presence of *Campylobacter* sp on chicken carcasses sold in retails. Almeida & Serrano (2) reported that 47.5% of chicken samples sold in Campinas (SP) carried *C. jejuni.* Leitão et al. (24), also in Campinas (SP), registered the presence of *C. jejuni/coli* in 62.2% of chicken carcasses acquired in butcheries of the region. Sakuma et al. (49), working in São Paulo (SP) with freshly eviscerated chicken products sold in retail outlets, found that 13.5% of the samples were contaminated with *C. jejuni/coli.*

In face of the importance of the genus *Campylobacter* as a human pathogen of great interest to the public health, the WHO Scientific Working Group on Epidemiology and Etiology of Enteric Infections called the attention of the scientific community upon the important role of foods in transmitting *Campylobacter* jejuni (57). In 1982, the Joint FAO/WHO Expert Committee on Food Safety included this microorganism in the roll of the etiological agents related to foodborne diseases (57).

Indirect epidemiological evidence has suggested that food products of animal origin, especially poultry products, constitute the main vehicle for human infection by *Campylobacter* species (6,20,33). Although not absolutely conclusive, the isolation of identical biotypes and serotypes from foods and human sources within the same studied area lends further support to the hypotesis of epidemiological correlation (47,51).

A study conducted by Harris et al.(15) on endemic enteritis due to *C. jejuni* revealed that poultry meat was responsable for about 48.2% of the campylobacteriosis cases, and 22.3% of the samples presented the same serotypes harboured by the affected persons.

In a recent survey carried out in the USA on foodborne diseases covering the period between 1973 and 1987, Bean & Griffin (5) concluded that *Campylobacter* species were the major etiological agents of bacterial diarrhea, and these infections were associated with eating chicken.

For all the reasons stated above, the present work was undertaken to obtain a profile of the occurrence of *Salmonella* and *Campylobacter* species on chicken during two steps of industrial processing.

MATERIALS AND METHODS

Chicken samples were collected during a 15 month period (1989-1990) in a processing plant of Santa Catarina State (Brazil) with a slaughtering capacity of around 25,000 units/day.

The slaughter, evisceration and packaging procedures in the processing plant were done manually.

Sampling Procedures

Samples were collected at two steps of the processing line. The first one just after stunning and slaughtering and the second after packaging in plastic bags (end product).

Immediately after slaughter, two samples were collected by swabing the cloacal region with cotton wool swabs and placed into test tubes (15 x 150mm) containing 10 ml of either of the following enrichment media for *Salmonella* sp: Tetrathionate broth (TTB-MERCK) or Rappaport-Vassiliadis broth (RV-Difco) (41,56). A similar procedure was used for *Campylobacter* sp using *Brucella* broth (Difco), supplemented with 0.15% agar (Difco), 0.15% bile salts (Difco), 5,0% defibrinated horse blood, FBP-solution and the antibiotics vancomicin, polymyxin and trimethoprin (Merck) (40).

The processed carcasses were first tested for *Campylobacter* sp by swabbing two superficial areas of the bird (back and cloacal region) using a sterile aluminium template (2cm x 5cm). Next, the swabs were immersed into 10ml *Brucella* broth plus supplements, as previously stated.

For *Salmonella* sp sampling, whole processed chicken carcasses were transfered to clean plastic bags containing 20 ml of buffered peptone water (9,31). After shaking and massaging the bags, each suspension was transfered to a sterile erlenmeyer and transported to the laboratory in ice-cooled containers. No more than 3 hours elapsed between sample collection and assaying.

Microbiological Assay

Salmonella sp

Inocula obtained from the first processing step in TTB and RV broth were incubated in a water-bath (FANEM) at 43°C for 24 hours; those from the second step collected in buffered peptone water were initially incubated in a bacteriological incubator (FABBE) at 35°C for 24 hours. Loopfuls of TTB an RV broths were streaked, respectively, onto Hektoen enteric agar (HEA-Difco) and brilliant green agar (BGA-Difco) and incubated at 35°C for 24 hours (41).

Five to six typical *Salmonella* sp. colonies grown on both selective media were tested in TSI and LIA slants (Difco) and then checked for the presence of urease and indol (-), the malonate test (-) and dulcitol fermentation (+). Serological tests were applied to suspected cultures using somatic and flagellar polivalent sera. (41). Complete serological indentification was done in a reference center (Instituto Oswaldo Cruz - Manguinhos, RJ, Brazil).

Campylobacter sp.

The swabs in *Brucella* broth were incubated at 42°C for 48 hours under microaerobic atmosphere, in an anaerobic jar (Permution do Brasil) fitted with a gas generator envelope (Anaerocult C-Merck) (40,54). These cultures were then streaked onto *Brucella*-FBP agar supplemented with 5% defibrinated horse blood and antibiotics and incubated at 42°C for 48 hours in modified atmosphere (40).

Typical colonies were selected by phase-contrast microscopic examination, purified by transfer to *Brucella*-FBP agar and *Brucella*-FBP broth and incubated at 42°C for 48 hours. The broth was incubated aerobically and the agar in microaerobic atmosphere. Presuntively identified cultures were confirmed by catalase and oxidase production, growth at 42°C but not at 25°C, nalidixic acid inhibition, cephalotin resistence, H_2S production, triphenyltetrazolium chloride tolerance and hypurate hydrolysis (29,40,54).

RESULTS AND DISCUSSION

A recent survey conducted in the USA by FSIS (USDA) in poultry processing plants showed that 3 to 5% of chickens arriving at the industry were positive for *Salmonella* sp, while 36% leaving it were contaminated with the microorganisms (13). To explain this, Lillard (27) suggested a possible cross-contamination during processing.

According to Table 1, the occurrence of *Salmonella* sp in the cloacal material of freshly slaughtered chickens was 10% and for the processed samples leaving the industry was 13.3%.

Results obtained by other investigators are very variable. A survey made in Canada by Higgins et al. (16) showed that the ocurrence of *Salmonella* sp in faecal samples collected from 6 week-old chickens varied from 0 to 88,8%. Using the cloacal swab procedure, Nivas et al (35) reported *Salmonella* sp levels of contamination

TABLE 1 - Occurrence of *Salmonella* on chicken carcasses examinated at two steps of industrial processing.

Sampling points	Sampling units		Mean (%)
	Examinated	Positives	
A	30	3	10.0
B	30	4	13.3

A - After slaughter; B - End product

between 1.3 and 16.4% while Barbour & Nabbut (4) found 7.4 and 27.6%. On the other hand, Jones et al (19) were not able to detect *Salmonella* sp in any of the chicken faecal samples examined.

It becomes evident, therefore, that some factors have to be taken into account concerning *Salmonella* detection, especially with regard to methodology and sample collection. According to Bailey & Cox (3), *Salmonella* recovery from poultry is more suitable when the sample is collected straightaway from the cecal region, because the cloacal procedure is not able to detect population levels below 10^4 organisms.

In this study, the occurrence of *Salmonella* sp on the end product, using the rinsing procedure (point B) was about 13.3% (Table 1). This value is lower than those found by others, like Rothschild Jr (48), Bokanyi Jr et al. (7), Machado & Bernardo (30) and Jones et al. (19) who reported contamination levels of 35.2%, 57%, 57% and 21.4%, respectively

Despite more than two thousand and two hundred serotypes of *Salmonella* being actually known and identified (25), only about twelve of them have been held responsible for cases of human salmonellosis (43), including *S. heidelberg* which is frequently isolated from processed chicken carcasses and cloacal material (19,28,48).

All the isolated cultures biochemically and serologically identified in this work were typed as *Salmonella heidelberg*.

Results of coprological examinations carried out on all the industrial workers involved in food processing were negative for *Salmonella* sp. Thus, the only possible explanation for the presence of *Salmonella heidelberg* on the end product was the initial contamination load of poultry arriving at the processing plant.

Regarding public health, these data need to be taken into consideration because *S. heidelberg*, together with a reduced group of other serotypes, are isolated rather often from human sources (8,21,34).

As to *Campylobacter* sp, a great deal of investigators agree that these microorganisms are part of the natural flora of poultry faeces and that during processing (especially during defeathering and evisceration) contamination of carcasses by faecal material can occur (1,12,18).

The microbiological analysis of 30 chicken samples just after slaughter (point A) and of 30 processed carcass surfaces (point B) revealed high incidence of *Campylobacter* sp with average values of 56.6% and 50.0%, respectively (Table 2). It was also observed that there was a remarkable predominance of *Campylobacter coli* over the other species, including *C. jejuni,* on both areas of the poultries examinated (Table 3).

TABLE 2 - Occurrence of *Campylobacter* on chicken carcasses at two steps of industrial processing.

Sampling points	Sampling units		Mean (%)
	Examinated	Positives	
A	30	17	56.6
B	30	15	50.0

A - After slaughter; B - End product

TABLE 3 - Distribution of *Campylobacter* species on chicken carcasses examinated at two steps of industrial processing.

Sampling points	Positives Cultures N°	*Campylobacter*							
		jejuni		*coli*		*laridis*		*NI*	
		N°	%	N°	%	N°	%	N°	%
A	23	3	13.0	13	56.5	3	13.0	4	17.4
B	18	1	5.5	14	77.8	1	5.5	2	11.1

A - After slaughter; B - End product

Our results are not in accordance with those found by other researchers. A great number of investigations has revealed a major occurrence of *C. jejuni* compared to *C. coli* during and after poultry processing (20,24,46,47). However, Sakuma et al. (49), analysing raw chicken samples purchased at a retail market in S. Paulo (Brazil), found that 86% of the positive samples were contaminated with *C. coli* and only 14% with *C. jejuni,* similarly to what was observed in our experiments.

With respect to the facts formerly referred, it is possible that immunological and genetic factors, as well as poultry dietary control during raising, play

an important role on the predominance of some enteric species over others, as has been observed by several investigators (17,22,38,39, 55).

In view of the stated, we can conclude that intestinal colonization appears to be the ultimate responsible for the contamination found throughout the processing line, as reported by Oosterom & De Wilde (37).

In conclusion, our data provide evidence that poultry is the apparently prominent reservoir of *Campylobacter* and *Salmonella* species. Such fact indicates the potential for transfer of these organisms to humans through consumption of contaminated chicken foods.

RESUMO

Ocorrência de *Salmonella* sp e *Campylobacter* sp em frangos durante o processamento industrial

Foi estudada a ocorrência de *Salmonella* sp e *Campylobacter* sp em frangos coletados em duas etapas (logo após o abate e também do produto final) de uma planta de processamento industrial de Santa Catarina. Nas amostras cecais examinadas logo após o abate, a positividade para *Campylobacter* sp e *Salmonella* sp foi, respectivamente, de 56.6 e 10.0%. Após o processamento das aves, as análises de superfícies para *Campylobacter* sp e enxague para *Salmonella* sp, revelaram níveis de positividade em 50.0 e em 13.3% das amostras, respectivamente. Também foi verificada uma maior presença de *C. coli* em relação a de *C. jejuni*, tanto nas amostras cloacais como nas de superfície. Por outro lado, foi encontrado apenas um sorotipo de *Salmonella* - *S.heidelberg* - em ambas as etapas da linha de processamento.

Palavras-chave: *Salmonella*, *Campylobacter*, frangos, processamento industrial.

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INOCULATION OF SOYBEAN IN CERRADO SOILS WITH ESTABLISHED POPULATIONS OF *BRADYRHIZOBIUM JAPONICUM*

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SUMMARY

The expansion of soybean producing areas in Brazilian Cerrados has promoted the establishment of populations of *Bradyrhizobium japonicum* in the soil; consequently, the benefit of reinoculation in those areas in now being questioned. The present study was conducted to measure the effectiveness of inoculation with selected strains on nodulation by the inoculated strains, when cultivated in soils containing different populations of *B. japonicum.* Serogroup 566 (USDA 123) was present in five of the six areas studied and was the dominant serogroup in three, although strains of that serogroup had never been used as an inoculant. Inoculation had no effect on total nodule numbers, except for one experiment, and no significant effect on grain yields. Inoculation with strains CPAC-7 and CPAC-15 increased the occurrence of their serogroups in soybean nodules to as much as 56%. That response was strongly reduced in soils where serogroup 566 was the dominant one. The data suggest that strains CPAC-7 and CPAC-15 can successfully overcome the native population for nodular infection sites, except in soils with a high incidence of serogroup 566.

Key words: reinoculation, nodulation, serogroup, serology.

INTRODUCTION

One fifth of Brazil (204 million hectares) constitutes an edaphic type of savanna known as "Cerrados" (2). With the development of economically viable fertilization practices, much of this area is becoming available for intensive agriculture. A rapid expansion of soybean production has occurred in the "Cerrados" and, as a result, a population of *Bradyrhizobium japonicum* has been established in soil, originally devoid of that bacterium (19). The benefit of continued soybean inoculation in those areas is now being questioned. Researchers in the U.S. (8, 10, 21) have reported that reinoculation of soybeans grown in soils with a history of soybean cropping has been notably unsuccessful in influencing nodulation or enhancing N_2 fixation above levels observed in uninoculated plants. The greater competitive ability of a strain of *B. japonicum* over another for nodulation sites is poorly understood. The selection of highly effective strain-cultivar pairings will not be practical until this knowledge is acquired. This limitation is a major obstacle to the development of inoculation procedures for introducing new strains of *B. japonicum* into fields with established population.

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The purpose of this study was to measure the effect of inoculation with selected strains of *B. japonicum* in soils previously cropped with inoculated soybean.

MATERIALS AND METHODS

Field experiments were conducted at six sites near Planaltina, Distrito Federal, Brazil in 1988 (experiment 1); 1989 (experiment 2); 1990 (experiments 3 and 4) and 1991 (experiments 5 and 6). The sites were well-drained oxisols classified as Dark Red Latosol (experiments 1 through 5) and Red Yellow Latosol (experiment 6). Except for one treatment in experiment three, inoculation was carried out at the level of 1 kg inoculant/400 kg of seeds. The treatments varied within the experiments. Experiment three consisted of the following treatments: a) Control, b) 29+587, c) CPAC-7, d) CPAC-15, e) CPAC-7+CPAC-15 and f) 400 kg N/ha. An additional treatment was included in that experiment, with strains CPAC-7+CPAC-15, at the level of 200 g/40 kg of seeds. The remaining experiments had only some of the above treatments.

Strain CPAC-15 was isolated from a Cerrado soil and gives a serological cross reaction with strain 566 (USDA 123); strain CPAC-7 (13) is a mutant derived from subcultures of strain CB 1809 (USDA 122). Strains 29W and 587 were isolated in Rio de Janeiro and Rio Grande do Sul, respectively, and have been used since 1980 in commercial inoculants manufactured in Brazil (13). Inoculants used in the experiments were prepared from agar slants of pure culture, in the laboratory of CPAC/EMBRAPA and had a bradhirizobial population varying from 7 x 10^7 to 3.4 x 10^8 cells/g, evaluated by the most probable number (MPN), using soybean as the host (20).

All sites had an established population of *B. japonicum* in the range of 10^3 to 10^4 cells/g soil (MPN). Plots had dimensions of 9x4m, 8x4m, 6x4m, 9x4m, 5x4m and 5x4m in the experiments 1, 2, 3, 4, 5 and 6 respectively and were planted at a rate of 25 viable seeds/m, with 0.50m between rows in the experiment 1, 5 and 6 and with 0.40m in the remaining ones. The soybean variety Doko was used in experiments 1, 2, 5 and 6, and variety Cristalina in experiments 3 and 4. Planting dates varied from November 15 through December 15 and harvesting dates were from April 30 through May 15.

Plant roots were collected 15 days after emergence (12 plants/plot) to evaluate the number of nodules. At the flowering stage, 6 plants per plot were collected for determination of number and dry weight of nodules and for serological analysis (20), using 40 nodules per plot in experiment 1 and 50 nodules in the remainig ones. Grain yields were determined in areas of 14m^2, 16.8m^2, 8m^2, 12,8m^2, 8m^2 and 8m^2 in experiments 1, 2, 3, 4, 5 and 6 respectively.

RESULTS AND DISCUSSION

Inoculation with *Bradyrhizobium japonicum* had no effect on number or dry weight of nodules except for experiment 4 at 15 days (Tables 1 and 2). Similar results were obtained in other papers (1, 8, 6, 3, 10, 19) in experiments carried out in soils with established populations of *B. japonicum.* In experiments 3, 5 and 6, fertilization with 400kg N/ha caused a decrease in the nodulation of plants evaluated at the flowering stage. Similar results were reported by Streeter (17).

TABLE 1 - Effect of reinoculation on nodulation of soybean at 15 days after plant emergency.

Treatments[a]	Experiments[c]					
	1	2	3	4	5	6
	number of nodules per plant					
Check	24	19	17	6b	32	27
29W + 587	25	19	17	10a	28	33
CPAC-7	24	15	16	9ab	-	-
CPAC-15	26	17	14	8ab	-	-
CPAC-7 + CPAC-15	-	18	15	-	32	26
200g / 40kg[b]	-	-	15	-	-	-
Nitrogen	-	-	14	-	29	32
Coef. of var. (%)	17	18	16	28	11	17

[a] All the inoculated treatment but one were treated with 1,000g inoculant/40kg of seeds.
[b] Inoculated with strains CPAC-7 + CPAC-15 at the level of 200g inoculant/40/kg of seeds.
[c] Values followed by the same letter do not differ statiscally by the Duncan test at 0.05.

The serological distribution of *B. japonicum* in soybean nodules is presented in Table 3. The data for the uninoculated treatment indicate the pattern for the occurrence of the strains in soil. This pattern varied for each area: in experiment 1, 5 and 6 strains of serogroup 566 (USDA 123)

TABLE 2 - Effect of reinoculation on the nodulation of soybean at flowering stage.

Treatments[1]	Experiments[3]					
	1	2	3	4	5	6
	number of nodules per plant					
Check	71	52	113a	44	98a	71ab
29W + 587	64	69	95a	60	89a	74ab
CPAC-7	49	47	97a	73	-	-
CPAC-15	59	58	123a	69	-	-
CPAC-7 + CPAC-15	-	69	112a	-	94a	88a
200g / 40kg[2]	-	-	102a	-	-	-
Nitrogen	-	-	51b	-	56b	59b
Coef. of var. (%)	27	31	27	34	18	17
	Dry weight of nodules (mg/plant)					
Check	183ab	107	204ab	159	218a	235a
29W + 587	191a	164	185b	232	185a	224a
CPAC-7	120b	126	206ab	220	-	-
CPAC-15	146ab	123	272a	231	-	-
CPAC-7 + CPAC-15	-	123	228ab	-	213a	252a
200g / 40kg++	-	-	189ab	-	-	-
Nitrogen	-	-	80c	-	88b	135b
Coef. of var. (%)	26	30	28	28	19	15

[1] All the inoculated treatment but one were treated with 1,000g inoculant/40kg of seeds.

[2] Inoculated with strains CPAC-7 + CPAC-15 at the level of 200g inoculant/40/kg of seeds.

[3] Values followed by the same letter do not differ statiscally by the Duncan test at 0.05.

were dominant in the nodules, whereas in experiment 3, 34% of the nodules did not react with any of the antisera used. This high percentage of nodules without reaction in that experiment was not expected; a preliminary evaluation of the same area seven months earlier showed the dominance (more than 81%) of serogroup 566 in uninoculated soybean nodules. An explanation for this result could be the instability of the somatic antigens in the bradirhizobial population, as suggested by Dudman (5). The percentages of nodules of serogroups 29W and 587 were less than 50% in four of the experiments, whereas in experiments 2 and 4 more than 70% of the nodules were of those serogroups. In those two experiments, the occurrence of serogroup 566 was very low (20% and 1%, respectively).

Even though serogroup 566 was found in the six areas, there is no record that strains of that serogroup had been inoculated in any of the experimental sites. Strain 566 was obtained from Dixie Inoculant (USA) and was used in Brazilian commercial inoculants until 1978. Since 1980, all the commercial inoculants have been based on strains 29W and 587. The occurrence of strains with serological reaction of strain 566 has been observed in several experimental areas and on soybean farms in Cerrados. The presence of strain 566 in soils where it apparently had never been inoculated may be due to its introduction with seeds or farm machinery from southern Brazil, where strain 566 was used for many years. In most soybean growing areas of northern midwestern USA, serogroup 566 (USDA 123) is dominant in soybean nodules regardless of the inoculated strains or the soybean variety (4, 8, 11, 21). The dominance of that serogroup in soils of Canada was also reported by Semu & Hume (16). There is as yet no acceptable explanation for the dominance of serogroup USDA 123 in those soils (11, 14).

Inoculation with strains CPAC-7 and CPAC-15 increased the participation of their serogroups in soybean nodules. That response varied according to the serological bradhirizobia composition of the soil; in experiments 1 and 3, where in the uninoculated control serogroups 29W and 587 it occurred in less than 50% of the nodules, inoculation with CPAC-7 increased significantly the participation of its serogroup to approximately 22%. In experiment 1, the increase in occurrence of serogroup CB 1809 in nodules was related to a decrease in the occurrence of serogroup 566, whereas in experiment 3 it was related to a decrease in serogroup 29W and a decrease in the percentage of non-reactive nodules (nodules that did not react with any of the antisera used). Inoculation with strain CPAC-15 (serogroup 566) promoted a small increase in the occurrence of its serogroup in soybean nodules. Since the soil also contained strains of serogroup 566, a more precise method is required to identify the inoculated strains and to quantify the actual participation of strain CPAC-15 in soybean nodules. It is worthwile to mention that in experiment 3, treatment inoculation with strain CPAC-15 gave a high percentage of non-reactive nodules (44%) that may have interferred from the interpretation of results.

In experiments 2 and 4, where more than 70% of the nodules in the uninoculated control was formed by serogroups 29W and 587, inoculation with either strain CPAC-7 or strain CPAC-15 resulted in a significant increase in the occurrence of their serogroups. By contrast, in experiments 1 and 3, serogroups 29W and 587 were found in less than 50% of the nodules in the uninoculated control and

TABLE 3 - Effect of reinoculation on serogroup distribution in soybean nodules.

Treatments[3]	Serogroups[1]									
	29W	587	566	CB-1809	n/r++	29W	587	566	CB-1809	n/r[2]
	%									
	Experiment 1					Experiment 2				
Check	19	12	66	0	3	28	49	20	3	0
29W + 587	12	16	70	0	1	35	45	11	1	8
CPAC-7	15	17	47	19*	2	14	25*	9	51*	1
CPAC-15	12	14	70	0	4	26	26*	46*	0	2
CPAC-7 + CPAC-15	-	-	-	-	-	22	31	37	9	1
Coef. of var. (%)	52	52	21	104	154	34	25	36	48	355
	Experiment 3					Experiment 4				
Check	34	15	16	1	34	33	66	1	1	0
29W + 587	32	33	9	2	24	33	62	1	0	4
CPAC-7	20	17	21	26*	16	16*	28*	0	56*	1
CPAC-15	15	19	21	0	44	18*	23*	58*	0	2
CPAC-7 + CPAC-15	31	12	26	11	19	-	-	-	-	-
200g / 40kg++++	28	15	15	5	37	-	-	-	-	-
Nitrogen	25	18	21	1	35	-	-	-	-	-
Coef. of var. (%)	35	55	51	82	57	30	22	39	61	123
	Experiment 5					Experiment 6				
Check	15	15	65	2	3	9	3	89	0	0
29W + 587	10	12	74	1	3	11	2	87	0	1
CPAC-7 + CPAC-15	10	14	65	11*	1	6	3	86	4*	1
Nitrogen	15	9	68	2	7	4	1	94	0	1
Coef. of var. (%)	44	37	11	65	73	64	106	6	94	207

1 Values followed by * are statistically different from the check by the Dunnett's test at p = 0.05.
2 n/r = no reaction with the antisera used.
3 All the inoculated treatment but one were treated with 1000g inoculant/40kg of seeds.
4 Inoculated with strains CPAC-7 + CPAC-15 at the level of 200g inoculant/40kg of seeds.

the response to inoculation was smaller. Inoculation with strain CPAC-7 increased the participation of its serogroup from 3 to 51% in experiment 2 and from 1 to 56% in experiment 4. Although the serological analysis did not distinguish the inoculated strain CPAC-15 from the soil strains of serogroup 566, the effect of inoculation can be seen in experiment 2, where participation of serogroup 566 increased from 20 to 46% for inoculation with strain CPAC-15. The increase in occurrence of serogroups of the inoculated strains in experiments 2 and 4 was related to a significant decrease in the occurrence of serogroups of the soil strains. Similar results were obtained by Kvien et al. (10) in soil with dominance of serogroup USDA 123; the response to inoculation varied according to the site, year and soybean genotypes.

Some of the factors affecting the inoculation response are the N_2-fixing efficiency (1, 15), competitiveness (8, 3, 10) and the size of the soil's indigenous bradirhizobial population (18). In this study, the results obtained with strains CPAC-7 and CPAC-15 indicate that the serological composition of the bradirhizobial population established in the soils is also a factor which affects the inoculation response of soybeans.

In experiments 2 and 3, co-inoculation with strains CPAC-7 plus CPAC-15 promoted average increases from 2 to 10% and from 18 to 31% in the occurrence of serogroups CB 1809 and 566, respectively. In experiments 5 and 6, sites where serogroup 566 was dominant, co-inoculation with strains CPAC-7 plus CPAC-15 did not promote an increase in the occurrence of serogroup 566. In

experiment 5, inoculation promoted an increase from 2 to 11% in the occurrence of serogroup CB 1809, whereas in experiment 6, although significant, that increase was only from 0 to 4%.

In experiment 3, co-inoculation with strains CPAC-7 plus CPAC-15 at the level of 200g inoculant/40kg seeds promoted an increase from 1 to 5% on the occurrence of serogroup CB 1809 in nodules, with no increase in the occurrence of serogroup 566; yet the percentage of nodules without reaction was very high (37%). That increase in the occurrence of the serogroup of the inoculated strain was lower than the ones obtained with a higher level of inoculant (1,000g/40kg of seeds); this indicates the importance of a higher concentration of rhizobial cells in the seeds to overcome the soil-born population, as observed by other investigators (9, 21, 3). In all experiments, inoculation with strains 29W and 587 failed to increase significantly the occurrence of their serogroups in soybean nodules.

Grain yields are presented in Table 4. In experiments 1, 3, 4 and 5, yield increases with inoculation varied from 81 to 417kg/ha depending on the strain used; however, these increases were not statistically significant. Kvien et al. (10) reported yield increases with soybean inoculation in soils with an established *B. japonicum* population even when the inoculated strain occurred at a low percentage in the nodules. In experiments 1, 2, 3, 4 and 5 inoculation and the use of N fertilizer had no effect on grain yield, indicating that the established soil bradirhizobial population was fixing N_2 at a level suficient to allow the development of the plants. In experiment 2, inoculation with strains CPAC-7 and CPAC-15 increased the participation of their serogroups in the nodules; however, no effect was also observed on grain yields, even though that strain was derived from CB 1809, which is know to increase grain harvest indexes even with lower nodule numbers (12).

TABLE 4 - Effect of reinoculation in the grain yield of soybean.

Treatments[1]	Experiments[3]					
	1	2	3	4	5	6
	Kg / ha					
Check	1933	3826	3575	2322	2127b	2241
29W + 587	1941	3497	3763	2589	2233b	2195
CPAC-7	2014	3799	3705	2612	-	-
CPAC-15	2034	3598	3744	2511	-	-
CPAC-7 + CPAC-15	-	3651	3961	-	2544b	2266
200g / 40kg[2]	-	-	3532	-	-	-
Nitrogen	-	-	3545	-	3109a	2265
Coef. of var. (%)	13	9	9	19	12	10

[1] All inoculated treatments but one were treated with 1000g inoculant/40kg of seeds.

[2] Inoculated with strains CPAC-7 + CPAC-15 at the level of 200g inoculant/40/kg of seeds.

[3] Values followed by the same letter do not differ statiscally by the Duncan test at 0.05.

RESUMO

Inoculação de soja nos cerrados com culturas estabelecidas de *Bradyrhizobium japonicum*

A expansão da área sob cultivo de soja nos cerrados brasileiros promoveu o estabelecimento de populações de *Bradyrhizobium japonicum*, sendo atualmente questionáveis os benefícios da reinoculação nessas áreas. O presente trabalho teve por objetivo avaliar a eficiência da inoculação com estirpes selecionadas, na nodulação, rendimento de grãos e participação das estirpes inoculadas nos nódulos de soja cultivada em solos com diferentes populações de *B. japonicum*. Sorogrupo 566 (USDA 123) estava presente em cinco dentre seis áreas estudadas, e foi o sorogrupo dominante em três delas, apesar desse sorogrupo nunca ter sido introduzido através de inoculação em nenhuma das áreas. Exceto por um experimento, a inoculação não afetou o número de nódulos das plantas e também não afetou significativamente os rendimentos de grãos. Observou-se que áreas inoculadas com as estirpes CPAC-7 e CPAC-15 apresentaram até 56% de ocorrência dos seus sorogrupos nos nódulos de soja. Essa resposta foi acentuadamente reduzida em solos com predominância do sorogrupo 566. Esses resultados indicam que as estirpes CPAC-7 e CPAC-15 podem superar as estirpes nativas de *B. japonicum* do solo na competição pelos sítios de infecção nodular, exceto em solos com alta ocorrência do sorogrupo 566.

Palavras-chave: reinoculação, nodulação, sorogrupo, sorologia, *Bradyrhizobium japonicum*.

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CELLULOLYTIC FUNGI ISOLATED FROM AN ALLUVIAL SOIL IN A SEMI-ARID AREA OF THE NORTHEAST OF BRAZIL

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SUMMARY

Soil borne fungi were isolated from an alluvial soil in a semi-arid (caatinga) zone of the State of Pernambuco, at a site with no vegetation which had been previously covered by "caatinga" species. The soil was caracterized as alic quartz-sandy with relatively high levels of Al^{+++} and an acidic pH. During the dry season, the soil's humidity is very low and its surface temperature can reach 70°C. One of the methods employed to determine soil water retention uses a dish of filter paper placed on a support set inside a plastic container filled with wet soil. Species of soil-borne fungi that grow and destroy these filter papers were isolated and identified. The ability of such species to attack paper is an indication of their cellulolytic activity. Two of the isolated species, namely: *Curvularia brachyspora* and *Penicillium pinophilum*, constitute new records for the State of Pernambuco.

Key words: cellulolytic fungi; soil; water retention; semi-arid; decomposition.

INTRODUCTION

Fungi are considered the major contributors to soil biomass and are known as an organotrophic group of species primarily responsible for the decomposition of organic matter (12). Among such species there are the filamentous ones and the yeasts, which actively participate in biodeterioration and biodegradation processes (1, 6), contributing to nutrient cycling and the preservation of ecosystems. The study and manipulation of soil microorganisms coupled to their metabolic processes can aid the improvement of crop productivity (8).

The characterization of fungal species able to degrade cellulose allows, through biochemical analysis, the detection of enzymes with industrial utility. The extracellular hydrolytic enzymes produced by fungi can degrade polymers of diverse carbohydrates, including cellulose (6), and are known as enzymes of the cellulase complex (1, 15); many species of common soil inhabiting fungi exhibit this enzymatic pattern.

Concerning soil studies, there are methods that may be affected by fungi present in the environment. This is especially true when cellulosic materials such as papers and cellulosic fibers are used during the analysis because cellulolytic fungi might impair the correct interpretation of the parameters studied.

The aim of the present investigation was to isolate soil fungi with cellulolytic activity which may contaminate filter papers used for some soil analyses.

MATERIALS AND METHODS

Soil samples were collected from a semi-arid zone (Petrolândia) of the State of Pernambuco

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(Brazil), during the dry season (November, December, January). The area, devoid of vegetation, had been previously covered by "caatinga" plant species. The soil was caracterized as alluvial (alic quartz-sandy soil) with a relatively high content of Al^{+++} and an acidic pH (Table 1). During the dry season, soil humidity is very low (1.5% and 3.3% at depths of 0.1 - 0.2m and 5.0 - 5.5m, respectively) and the soil surface temperature reaches 70°C.

TABLE 1 - Chemical caracteristics and granulometric composition of the alluvial soil from a semi-arid area of the State of Pernambuco.

Depth (m)	Al^{+++} Meq/ 100 g 0	pH H_20	P ppm	N %	C %	O. M. %	M. S. %	F. S. %	Silt %	Clay %
0.0 - 0.1	0.40	5.0	2.80	0.06	0.36	0.62	-	-	-	-
0.2 - 0.3	0.55	4.8	1.22	0.04	0.19	0.33	20	76	2	2
0.4 - 0.5	0.75	4.6	1.45	0.04	0.24	0.41	24	71	3	2
0.6 - 0.7	0.75	4.3	0.88	0.04	0.15	0.26	21	76	1	2
0.8 - 0.9	0.75	4.4	0.77	0.03	0.20	0.34	18	76	2	4
1.0 - 1.1	0.90	4.4	0.77	0.03	0.21	0.36	18	75	1	6
1.2 - 1.3	0.85	4.3	0.77	0.04	0.15	0.26	24	68	2	6
1.4 - 1.5	0.90	4.3	0.77	0.04	0.20	0.34	18	76	1	5
1.6 - 1.7	0.85	4.3	0.55	0.04	0.12	0.21	16	78	2	4
1.8 - 1.9	0.75	4.2	0.43	0.04	0.15	0.26	18	74	2	6
2.0 - 2.5	0.70	4.5	0.55	0.04	0.18	0.31	17	75	2	6
2.6 - 3.0	0.70	4.5	0.55	0.03	0.16	0.28	16	75	3	6
3.1 - 3.5	0.55	4.5	0.55	0.03	0.15	0.26	17	74	3	6
3.6 - 3.9	0.45	4.5	0.88	0.04	0.13	0.22	19	72	2	7
4.0 - 4.5	0.40	4.6	0.32	0.03	0.16	0.28	15	75	2	8
4.6 - 5.0	0.25	5.0	0.32	0.04	0.17	0.29	17	72	6	5
5.1 - 5.5	0.35	5.1	0.55	0.03	0.13	0.22	19	70	3	6

O. M. = organic matter; M. S. = medium sand; F. S. = fine sand

Samples were collected at different soil depths and placed inside separate plastic containers for chemical and granulometric analysis as well as evaluation of water retention. Each container (9cm high X 6cm diam) had a plastic support covered by a filter paper dish. Comparison of the humidity retained by the filter paper allows the determination of the soil 's water retention (10). Dishes of filter paper colonized by fungi in the containers were transferred to sterilized Petri dishes and maintained in a humid chamber. Subsequently, samples of each one of the developed fungal colonies were transferred to Petri dishes containing specific medium (Sabouraud plus antibiotic). Inocula of the newly developed colonies were transferred to the same type of medium or to other media (potato-dextrose-agar; malt agar) for further identification based on the specialized literature (5, 7, 13, 14). Samples of filter paper taken from the same box used for soil analysis were used as contaminant-free controls. These samples were placed in humid chambers and observed for a period of ten days.

RESULTS AND DISCUSSION

Twenty two species of filamentous fungi were isolated from the dishes of filter paper used for the analysis of soil water retention potential (Table 2). The results probably reflect the isolation of species which can disseminate faster, as opposed to slow-growing species or those less able to pass from the soil to the filter paper inside the container. Some species of *Cladosporium, Fusarium, Aspergillus,* and *Penicillium,* for example, were isolated from soil but were not detected on the filter paper dishes (data not published). Results from the filter paper samples used as control were negative, indicating that they were free of contamination.

No method for the isolation of soil fungi is one hundred percent efficient. In the present investigation, the only surface to be colonized by fungi was the filter paper dish and this might have restricted the isolation of these microorganisms to exclusively cellulolytic species. Another factor to be considered regarding the amount of isolated species is the fact that microbial biomass is directly correlated with the amount of vegetation

TABLE 2 - Species of fungi isolated from filter paper maintained inside the containers filled with wet soil used for the analysis of soil water retention potential.

Species*	Soil depth (m) 0.0-0.5	0.6-2.0	3.0-4.5	5.5-6.5	7.0-9.5
Alternaria alternata			X	X	
Aspergillus deflectus		X	X	X	X
Aspergillus japonicus		X			
Aspergillus niger		X			
Aspergillus versicolor				X	
Aspergillus ustus		X			
Aspergillus sidowii		X			
Chaetomium globosum		X	X		
Curvularia brachyspora	X				
Curvularia pallescens			X		
Gliocladium sp.		X			
Humicola fuscoatra	X				
Penicillium funiculosum		X			
Penicillium pinophilum	X				
Penicillium puberulum		X			
Penicillium verruculosum	X				
Penicillium walksmanii		X			
Pithomyces chartarum		X	X	X	
Rhizopus oryzae					X
Scolecobasidium constrictum					X
Trichoderma harzianum		X			
Trichoderma pseudokoningii			X		

* Cultures maintained at URM (Departamento de Micologia - CCB - UFPE):
A. alternata (Fr.) *Keissler* (3298); *A. deflectus* Fennell & Raper (3294); *A. japonicus* Saito (3289); *A. niger* van Tieghem (3292); *A. versicolor* (Vuill.) Tiraboschi (3293); *A. ustus* (Bain) Thom & Church (3291); *A. sidowii* (Bain. & Sart.) Thom & Church (3290); *C. globosum* Kunze ex Steud (3304); *C. brachyspora* Boedijn (3297); *C. pallescens* Boedijn (3296); *H. fuscoatra* Traaen (3300); *P. funiculosum* Thom (3287); *P. pinophilum* Hedgcock (3285); *P. puberulum* Bain. (3295); *P. verruculosum* Gilman & Abbott (3288); *P. walksmanii* Zaleski (3286); *P. chartarum* (Berk & Kurt); M. B. Ellis (3301); *R. oryzae* Went & Prinsen Geerl. (3310); *S. constrictum* Abbot (3299); *T. harzianum* Rifai (3302); *T. pseudokoningii* Rifai (3303).

that covers the soil surface and the level of soil organic matter. Most of the species were found in layers close to the soil surface, where the amount of organic matter is generally higher (12). However, since the soil studied had no vegetation cover, the amount of organic matter present was small. Moreover, the average soil humidity at the soil surface and at deeper layers was extremely low. The major factors affecting fungal activity are temperature and water content of the substrate (4); considering that the analysed soil was caracterized by low humidity and high temperature, the fact that only a few species of fungi were isolated is not surprising.

The results indicate that a greater species diversity exists at soil depths of up to 2 meters in the environment studied (Table 2). In general, the amount of soil microorganisms and organic matter decreases with soil depth. However, population density does not decrease to extinction and fluctuations occur in the lower layers of soil. In alluvial soils, microbial populations fluctuate according to changes in soil texture and the organisms are more frequently found on silty rather than on sandy layers (12). The alluvial soil studied has a relatively uniform granulometric constitution, with a small increase in the amount of silt below 0.8m of soil depth (Table 1). Thus, species density was probably related to other soil factors.

The decomposition of cellulose is one of the most important processes of organic matter degradation promoted by fungi. Most of the isolated species are referred to as cellulolytic and some have been used as a source of enzymes of the cellulosic complex (Table 3).

TABLE 3 - References on the cellulolytic activity of the isolated taxa.

Genus / species	References
Alternaria alternata	5, 7
Aspergillus spp	5, 11
Chaetomium globosum	1, 5, 7, 9
Curvularia spp	2, 3
Gliocladium spp	1, 5, 7, 9
Humicola fuscoatra	1, 5, 7, 9
Penicillium spp	9
Pithomyces chartarum	7
Trichoderma spp	5, 9, 11

Alternaria alternata is the most common species of the genus; occurring on plants, soils, and on many others substrates, it has been largely known as a biodegradation agent (5). Most of the species of *Aspergillus* are capable of promoting cellulose breakdown (5) and *A. niger* has been used as source of cellulolytic enzymes (11). References to *Curvularia* species as cellulose decomposers are relatively new (2, 3). Two species were found on the dishes of filter paper, which confirms their cellulolytic potential. *Gliocladium* spp., *Humicola fuscoatra* and *Chaetomium globosum* are common soil-inhabiting fungi of which the ability to degrade cellulose has been extensively documented (1, 5, 7, 9). *Pithomyces chartarum*, species of *Trichoderma* and of *Penicillium* are known as active cellulose decomposers (7, 9), and some are primary source of cellulolytic enzymes (11). The ability of *Rhizopus oryzae* and *Scolecobasidium constrictum* to degrade cellulose was shown by the growth of their colonies on the filter paper dishes.

Among the species isolated, two constitute new records for the State of Pernambuco, namely: *Curvularia brachyspora* and *Penicillium pinophilum*. This is also the first report of the isolation of such species from an exclusively cellulolytic substrate.

Considering that the presence of fungi on dishes of filter paper used for the analysis of soil water retention may define the method's unviability, the use of different techniques should be enforced, employing materials not susceptible to easy microbial contamination.

The cellulolytic species isolated might be used for further application in the industry, for the production of enzymes of the cellulase complex, and for quality control tests of diverse manufactured materials such as paper and its secondary products.

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RESUMO

Fungos celulolíticos isolados de solo aluvial numa zona semi-árida do nordeste do Brasil

Fungos foram isolados de um solo aluvial numa zona semi-árida (caatinga) do Estado de Pernambuco, em área sem vegetação, anteriormente coberta por espécies de caatinga. O solo foi caracterizado como álico quartzo-arenoso com níveis relativamente altos de Al^{+++} e pH ácido. Durante a estação seca a umidade do solo é muito baixa e a temperatura na superfície pode chegar a 70°C. Um dos métodos para determinar a retenção de água pelo solo inclui o uso de um disco de papel de filtro colocado sobre um suporte, dentro de um recipiente plástico contendo solo úmido. Fungos do solo que crescem e destroem o papel de filtro foram isolados e identificados. A capacidade dessas espécies para atacar o papel indica a sua atividade celulolítica. Duas das espécies isoladas *(Curvularia brachyspora e Penicillium pinophilum)* constituem novas referências para o Estado de Pernambuco.

Palavras-chave: fungos celulotíticos, retenção de água, semi-árido, decomposição

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BACILLUS SUBTILIS AS A POTENTIAL BIOCONTROL AGENT OF THE NORTHERN LEAF BLIGHT OF CORN

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SUMMARY

Four *Bacillus subtilis* isolates (AP-3, AP-420, IF-36 and IF-82) were studied as potential agents for the biocontrol of the Northern leaf blight of corn, caused by *Exserohilum turcicum*. The bacteria demonstrated good biocontrol efficiency under greenhouse conditions. Considering the average value of the three application periods, AP-420 proved to be the most effective. Application of the antagonists at the same time as *E. turcicum* inoculation gave the best results. Under laboratory conditions, the bacteria produced extracellular, non-volatile and difusible metabolites which inhibited *E. turcicum* mycelial growth and conidial germination.

Key words: *Exserohilum turcicum*, corn, biocontrol, *Bacillus subtilis*, Northern leaf blight.

INTRODUCTION

Northern leaf blight, caused by the fungus *Exserohilum turcicum* (Pass.) Leonard & Suggs (syn = *Helminthosporium turcicum* Pass.), is an important disease of corn (*Zea mays* L.) (29). The symptoms are necrotic leaf blights which reduce the photosynthetic area (3). Depending on the time of disease onset and presence of adequate moisture, yield losses as high as 68% can occur (25). Disease control is mainly performed by resistant cultures and fungicide applications (3, 29). However, the variable efficiency of the chemical control has been reported (9). Nowadays, biological control is an important option for the management of some plant diseases (8). The use of bacteria as controlling agents has been shown to be a strategy of great potential (27, 32). One of the most studied antagonists of the foliar plant pathogens is the bacterium *Bacillus subtilis* Cohn. Its ability to reduce fungal plant diseases is related to its strong production of inhibitory antibiotics and extracellular metabolites (30).

Considering the economic importance of the disease and the variable efficiency of the traditional control metholds, this work aimed to study the potential of some *B. subtilis* isolates as biocontrol agents of Northern corn leaf blight.

MATERIALS AND METHODS

Microorganisms

The *E. turcicum* isolate (HT-18) was obtained from infected corn leaves collected

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from a commercial field, in Serra Talhada-PE. Four *B. subtilis* isolates (AP-3, AP-420, IF-36 and IF-82), considered good antagonists to other air-borne plant pathogens in previous studies (4, 21), were obtained from the stock collections of microorganisms of the Laboratório de Fitobacteriologia (Departamento de Agronomia - UFRPE, Recife-PE, Brasil), and the Laboratório de Fitopatologia (Centro Nacional de Pesquisa de Defesa da Agricultura - EMBRAPA, Jaguariúna-SP, Brasil).

The microorganisms used in the experiments were cultures as follows: The *E. turcicum* matrix was grown for 12 days on corn extract-agar medium - CEA (31) in the dark; *B. subtilis* was grown on nutrient-yeast-dextrose-àgar médium - NYDA (28) for 48 hs.

Greenhouse experiment

B. subtilis isolates were evaluated for their ability to reduce the severity of the Northern leaf blight of corn.

Greenhouse-grown 3 week-old corn plants, cv. Centralmex, were inoculated with the *E. turcicum* suspension ($5x10^3$ conidia/ml) and treated with a bacterial suspension (10^8 cfu/ml). The fungicide mancozeb (Dithane PM, 800g a.i./kg, Rohm and Haas Brasil Ltda, Brazil) was used as a standard treatment at 1,000 ppm a.i. All suspensions were amended with 0.05% Tween 80 in water. Sprays were performed until runoff using a De Vilbiss atomizer. Antagonists and fungicide were applied 2 days before, at the same time as or 2 days after the inoculation of the pathogen. Control plants were inoculated with *E. turcicum* but received no bacteria or fungicide. Following inoculation, plants were placed for 18 hs in a moist chamber (30 ± 2°C and 98,5% humidity) and then returned to the greenhouse (31 ± 2°C and 87% humidity).

Disease severity was evaluated 10 days after inoculation by counting the number of lesions in three leaves per plant. The percentage of disease severity reduction (DSR) was calculated according to the formula: DSR (%) = [(LC - LT)/LT] x 100, where LC = lesion number from the control plants and LT = lesion number from the treated plants (21).

Laboratory experiments

The ability of *B. subtilis* isolates to produce inhibitory substances against *E. turcicum* was evaluated using the following methods: paired cultures, production of non-volatile metabolites, and paired suspensions (20).

Paired cultures

Two agar disks (6 mm in diameter) from cultures of *E. turcicum* were placed 70 mm apart on a Petri dish containing CEA medium. After 12 hr incubation, each bacterial isolate was streaked as a band in the center of a plate, except for the controls.

Production of non-volatile metabolites

A loopfull of the bacterial growth was centrally placed on sterile cellophane membrane over CEA medium inside Petri dishes. The membrane was removed after 96 hr and a 6mm disc of *E. turcicum* culture was placed in the center of the plate. Controls did not receive the bacteria.

With both methods (paired cultures and production of non-volatile metabolites), plates were incubated under alternate light (12 hr light/12 hr dark) at 25±2°C and 70% humidity; they were evaluated 10 days later by measuring the pathogen's linear mycelial growth. The percentage of mycelial growth inhibition (MGI %) was calculated according to the formula: MGI (%) = [(MGC -MGT)/MGC] x 100, where MGC = mycelial growth on the control and MGT = mycelial growth on the treatment (12).

Paired suspensions

Droplets (0.2 ml) of sterile distilled water containing *E. turcicum* ($5x10^3$ conidia/ml) and test bacterium (10^8 cfu/ml) or mancozeb (1,000 ppm a.i.) were placed inside the cavity of glass slides. Controls did not receive either bacterial or fungicide suspensions. After an incubation period of 7 hr in dark at 25±2°C, a drop of cotton blue (1%) was placed into each cavity and the number of germinated conidia counted under a light microscope (X100 magnification). A conidium was considered germinated when the length of the germ tube was twice its largest width. The percentage of germinated conidia was given by the average of five fields for each replication. The percentage of conidia germination inhibition (CGI) was calculated according to the formula: CGI (%) = [(CGC - CGT)/CGC] x 100, where CGC = conidia germination on the control, and CGT = conidia germination on the treatment (12).

The results were subjected to an analysis of variance (ANOVA), followed by Tukey's test using SANEST software (Instituto Agronômico de Campinas-IAC, Campinas-SP, Brasil).

RESULTS

B. subtilis isolates showed good efficiency biocontrol agents of Northern leaf blight of corn when evaluated 10 days after *E. turcicum* inoculation (Table 1). Considering the average of the three application periods, AP-420 showed the best disease severity reduction (44.6%),which, however, was not significantly different from that obtained by chemical treatment (42.7%).

There was a significant difference in the disease severity reduction (DSR) when comparing values from the various antagonist application periods. Best results were obtained when application of the antagonists was carried out concomitant to the inoculation of the pathogen: in this case, the highest value was observed for IF-82 (57.6% DSR) though not differing significantly from the results obtained with AP-420 ((56.7% DSR), mancozeb and other isolates. The application of AP-420 2 days before *E. turcicum* inoculation also provided good results, with 51.6% of disease severity reduction. IF-82 showed less efficiency when applied 2 days after pathogen inoculation.

Under laboratory conditions, the paired culture test showed that all *B. subtilis* isolates inhibited *E. turcicum* mycelial growth (Table 2), with formation of inhibition zones. AP-3 was the best antagonist, inhibiting the mycelial growth by 37.1% and differing significantly from the other isolates.

All bacteria produced extracellular non-volatile metabolites which inhibited *E. turcicum* mycelial growth (Table 2). The IF-82 metabolite(s) showed a better inhibitory effect (38.2%), which, nonetheless, was very close to that observed for AP-3 (36,0%)

E. turcicum conidium germination was significantly affected by both bacteria and fungicide suspensions (Table 3). In the absence of bacteria or mancozeb, conidia of *E. turcicum* produced long and polar germination tubes after 7 hr of incubation in sterile distilled water. Results from the paired suspension test indicate that bacteria induced the inhibition and malformation of the pathogen's germ tubes which became short, bulbous, with rudimentary primary branching. IF-82 was by far the best bacterial antagonist (77.8%). Nevertheless, mancozeb induced the highest inhibibition of conidial germination (97.7%).

TABLE 2 - Mycelial growth inhibition of *Exserrohilum turcicum* induced by *Bacillus subtilis* according to paired culture (PCT) and productin of non-volatile metabolites (PNM) tests.

Bacterial Isolated	Micelial growth inhibition (%)[1]	
	PCT	PNM
AP-3	37.1 a	36.0 a
IF-36	33.5 b	27.9 b
IF-82	33.0 b	38.2 a
AP-420	31.2 c	26.2 b

[1] average of 6 replicates. Means followed by the same letter do not differ (p = 0.05) according to Tukey's test.

TABLE 1 - Influence of the *Bacillus subtilis* application periods on Northern corn leaf blight severity reduction (DSR%) 10 days after *Exserohilum turcicum* inoculation.

Application period	DSR(%)[1]	Treatment	DSR(%)[2]	Treatment X Application period - DSR (%)[3]			
				Treatment	-2	0	+3
0	54.4 a	AP-420	44.6 a	AP-420	51.6 a A	56.7 a A	25.6 a B
-2	39.4 b	mancozeb	42.7 a	mancozeb	44.6 ab B	54.8 a A	28.8 a C
+2	29.2 c	IF-36	40.4 ab	IF-36	39.7 bc B	52.2 a A	29.2 a C
		AP-3	35.6 bc	AP-3	29.6 c B	50.8 a A	26.4 a B
		IF-82	34.3 c	IF-82	31.7 cd B	57.6 a A	13.5 b C

[1] Average of 15 replicates

[2] Average of 9 replicates

[3] Average Of 3 replicates

Means followed by the same letter (lower-case letters for rows and upper-case letters for columns) do not differ (p=0.05) according to Tukey's Test.

Application periods in relation to time of pathogen inoculation: -2 = two days before; 0 = at the time as; + 2 = two days after.

TABLE 3 - Conidial germination inhibition of *Exserohilum turcicum* by *Bacillus subtilis* and mancozeb evaluated by the paired suspension test.

Treatment	Conidial germination inhibition (%)
mancozeb	97.7 a
IF-82	77.8 b
IF-36	67.1 c
AP-3	52.7 d
AP-420	43.6 e

[1] Average of 6 replicates. Means followed by the same letter do not differ (p = 0.05) according to Tukey's test.

DISCUSSION

Northern corn leaf blight control through the application of *B. subtilis* at the same time as pathogen inoculation has better efficiency than the applications before or after the pathogen inoculation. This agree of with the results obtained for the biocontroling *Alternaria alternata* (Fr.) Keissl. with *B. subtilis* on tobacco *(Nicotiana tabacum* L.) (16). Considering the levels of biocontrol through bacterial applications (23, 32), the results presented in this study can be taken as very significant. The efficiency of bacterial treatments is probably related to the behaviour of *E. turcicum* during its pre-penetration development on the host surface, as reported by Fokkema (14) for other necrotrophic leaf pathogens.

The *B. subtilis* isolates showed little efficiency on the control of northern leaf blight of corn when applied after the pathogen inoculation. This suggests the bacterium has low colonization and competition abilities following pathogen establishment, and/or that the pathogen exhibits low vulnerability to the antagonists once established on the host.

E. turcicum mycelial growth was inhibited under laboratory conditions as shown by the paired culture and production of non-volatile metabolites tests. This demonstrates the fungistatic property of *B. subtilis* isolates and characterizes the antibiosis mechanism discussed by Cook & Baker (10), which is usually associated with lethal action or chemical toxicity. Similar results were observed for the interactions of *B. subtilis* with *E. turcicum* (13) and other phytopathogens (13, 23, 30).

The fast germination exhibited by *E. turcicum* in sterile distilled water indicates its low dependence on exogenous nutrients to form infection structures, as reported by Blakeman (6) for other necrotrophic leaf pathogens. Leach *et al.* (18) observed that *E. turcicum* conidium germination before 6 hr and the mature development on corn leaf lesions occurred after 12 hr in darkness.

Similar observations on the formation of long and polar germ tubes on *E. turcicum* conidia obtained in the present study has also been described by Levy & Cohen (19).

The inhibitory effects of *B. subtilis* isolates on the *E. turcicum* conidium germination, under laboratory conditions are similar to those obtained through the use of *Bacillus* spp. against *H. sativum* (Pam.) King & Bakke (17), *Bipolaris oryzae* (Breda-de-Haan) Shoem. (23), and other necrotrophic (4, 21) and biotrophic leaf pathogens (5, 22). The abnormalities observed in *E. turcicum* germ tubes when paired with bacterial cells are similar to those described by Nanda & Gangopadhyay (23) following interactions between *B. subtilis* and *B. oryzae.*

The antagonic effect of *B. subtilis* isolates on conidium germination of *E. turcicum* is probably related to the production and diffusion of extracellular non-volatile metabolites. Antibiosis demonstrated for *B. subtilis* spore germination of pathogenic fungi is considered significant in disease biocontrol (4, 21, 23). However, an instability of antibiotics and metabolites produced by microorganisms in phylloplane has been reported and described as caused by adsorption, imobilization, oxidation, dilution and repeated condensation as well as by water evaporation (7).

B. subtilis isolates varied in the ability to inhibit growth of *E. turcicum* and/or to control the Northern leaf blight of corn. This might be due to genetic variability or to environmental factors (1).

The antifungal activity "in vitro" does not always correspond to disease reduction "in vivo" (1, 2, 8). In the present study *B. subtilis* AP-420 showed a less pronounced antagonistic effect against *E. turcicum* under laboratory conditions than under greenhouse condition, being the best biocontrol agent in the latter case. This may reflect the fact that antagonisms against *E. turcicum* involved mechanisms not detected by "in vitro" tests, such as nutrient and/or space competition and induced resistance. This was also verified in some phylloplane interactions (11,14) since it is unlikely that a single mechanism is responsible for the total biocontrol activity (24).

The present study suggests that AP-420 has good potential as a biocontrol agent against Northern leaf blight of corn.

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RESUMO

Potencial de *Bacillus subtilis* como agente de biocontrole da queima das folhas do milho

Quatro isolados de *Bacillus subtilis* (AP-3, AP-420, IF-36 e IF-82) foram estudados como agentes potenciais para o biocontrole da queima das folhas do milho, causada por *Exserohilum turcicum*. Em condições de casa-de-vegetação os isolados bacterianos demonstraram boa eficiência para o biocontrole. Considerando a média de três períodos de aplicação, o isolado AP-420 foi o mais eficiente. A aplicação dos antagonistas simultaneamente com a inoculação de *E. turcicum* apresentou os melhores resultados. Em condições de laboratório as bactérias produziram metabólitos extracelulares, não-voláteis e difusíveis que inibiram o crescimento micelial e a germinação de conídios de *E. turcicum* .

Palavras-chave: *Exserohilum turcicum*, milho, biocontrole, *Bacillus subtilis*, queima das folhas.

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FORMULATION OF CULTURE MEDIUM FOR GROWTH OF MICROBES FROM THE SUGAR CANE INDUSTRY AND USE OF CETYLTRIMETHYLAMMONIUM BROMIDE AS ANTIMICROBIAL AGENT

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SUMMARY

A culture medium appropriate for the cultivation of microorganisms relevant to the sugar cane industry was formulated. Medium efficiency was determined according to growth parameter measurements taken for the major cane juice biocontaminants cultured, known to be seletive in their growth factor requirements. Medium formulation was based on the composition of sugar cane juice, since it was intended for *in vitro* evaluation of the effectiveness of antimicrobial agents commonly used against troublesome microbial contaminants from the sugar cane industry. To test the usefulness of the medium, the activity of cetyltrimethylammonium bromide (CTAB) on some selected microorganisms was investigated. Acetic acid bacteria and lactic acid bacteria were the most sensitive to CTAB, followed by yeast, *Pseudomonas acidovorans* and enterobacteriaceae. Some *Pseudomonas* species (*Ps. aeruginosa, Ps. cepacia and Ps. mendocina*) were extremely resistant to CTAB, probably due to their cell envelope impermeability. Using the presently described technique, it was possible to measure the effectiveness of CTAB at very low concentrations on microorganisms important for the sugar cane industry.

Key words: bacteriostatic agent; antimicrobial test; sugar industry; cetyltrimethylammonium bromide.

INTRODUCTION

Antimicrobial agents are frequently used during the production of alcohol and sugar from sugar cane, to reduce damage caused by naturally occuring bacteria and fungi. During the sugar cane washing process, lime (calcium hydroxide) is frequently added to wash water to bring its alkalinity near pH 10,5 so as to inhibit microbial proliferation. Several combinations of antimicrobial compounds are added to the crushing and extraction systems to decrease the growth of microbes on the extraction equipment (32,23). During sugar cane alcoholic fermentation, bacterial contaminants may affect yeast activity, reducing fermentation yield and productivity and also increasing the acidity of the alcohol produced (29). These contaminants are normally controlled by treatment of pitching yeast with sulfuric acid and antibacterial agents (2). For economic reasons, the concentrations of antimicrobial agents used in the sugar cane industry are, in general, much lower than those normally employed for the disinfection of materials. Hence, methods currently employed to determine the bactericidal effect of antimicrobial compounds, such as the

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phenol index (14), are of limited value for testing the compound's actual effectiveness during sugar cane industrial processing. Even the measurement of a bacteriostatic effect such as the minimal inhibitory concentration (m.i.c.) does not evaluate the effectiveness of antimicrobial agents during industrial aplications, because the amount of agent applied in such cases is usually much less than required to reach m.i.c. (23). Actually, the main aim of the use of antimicrobial agents in sugar cane industrial processes is to reduce both the growth and the proliferation rate of contaminants, so that microbial activity no longer affects normal industrial operations in a significant manner. It is thus important to develop a laboratory technique that measures partial inhibition of microorganisms by antimicrobial agents applied in the manufacturing process thereby indicating more accurately the actual effectiveness of such compounds in industrial applications (30,5).

The results presented in this work were obtained with the tecnique developed for measuring antimicrobial activity according to the reduction of microbial growth rate. Firstly, a culture medium which permitted fast growth of species known as major contaminants of sugar refining processes was developed. Obviously, its formulation had to be based on sugar cane juice, since the effectiveness of cetyltrimethylammonium bromide (CTAB), a compound chosen for being a common component of antimicrobial formulations applied in the sugar industry (33), was evaluated.

The growth of microbes is normally measured by the following parameters: specific growth rate (μ); lag time (L) and maximum cell density (C_M). The maximum cell density has limited meaning in this study because, in normal industrial operations, the substrate is found in large excess and contaminants are usually well below C_M. Conversely, the parameters μ and L are more important because they show actual growth of contaminants permitting an easy detection of the effect of several interfering factors on microbial development.

The lag time (L), defined by Lodge & Hinshelwood (16) as the time required for a microbe to reach exponencial growth, was replaced by lag factor (R) proposed by Ibarra-Leon & Yokoya (13) in this work because: a) it is dimensionless and normally ranges between zero and 1, and b) it is less affected by factors that act on both lag time and growth rate, such as temperature of incubation and pH of medium. The lag factor is defined as:

$$R = \frac{C_p}{C_o}$$

where Co is the inicial cell concentration and Cp is the extension of exponential growth curve to initial time (t_o).

Several microbes were tested, mainly those found in sugar cane industry operations. They were yeasts and Gram positive and Gram negative bacteria.

MATERIALS AND METHODS

Cetyltrimethylammonium bromide (CTAB) purity 99% was purchased from Sigma Chemical Co.

Culture media: The MRS medium (proteose peptone 10 g/l, beef extract 10 g/l, yeast extract 5 g/l, dextrose 20 g/l, ammonium citrate 2 g/l, dissodium phosphate 1 g/l, sodium acetate 5 g/l, magnesium sulphate 0.1 g/l, manganese sulphate 0.05 g/l, Tween 80 1 g/l) was used for growth and maintenance of *Lactobacillus fermentum*, *Leuconostoc mesenteroides* and *Klebsiella terrigena;* the ACE medium (tryptone 50 g/l, yeast extract 2 g/l, dipotassium phosphate 1 g/l, dextrose 20 g/l) was used for growth and maintenance of *Acetobacter* species.

Microorganisms: *Acetobacter aceti* IZ 2023 and *A. pasteurianus* IZ 2024 were obtained from the culture collection of Escola Superior de Agricultura "Luíz de Queiróz", Piracicaba-S.P. *Lactobacillus fermentum* FTPT 0694, *Leuconostoc mesenteroides* FTPT 0367, *Klebsiella terrigena* FTPT 0400, *Escherichia coli* FTPT 0923, *Saccharomyces cerevisiae* FTPT 0472, *Pseudomonas aeruginosa* ATCC 10145, *Ps. mendocina* ATCC 25411, *Ps cepacia* ATCC 25416, *Ps. acidovorans* ATCC 17438 and *Alcaligenes faecalis* ATCC 8750 were obtained from the culture collection of Fundação Tropical de Pesquisa e Tecnologia André Tosello, Campinas -S.P.

Growth of bacteria for media formulations: Active cultures of *Lactobacillus fermentum*, *Leuconostoc mesenteroides* and *Klebsiella terrigena* were inoculated in spectrophotometer tubes (sterilized with 70% ethanol); sterile test medium was then added and the tubes were incubated at 30°C. *Acetobacter aceti* e *A. pasteurianus* were inoculated in 100 ml erlenmeyer flask with 20 ml medium and

TABLE 1 - Media formulations with sugar cane juice.

Added chemicals	Media										
	1	2	3	4	5	6	7	8	9	10	11
A	100	100	100	100	100	100	100	100	100	100	100
B	5	5	5	5	5	5	5	5	5	5	5
C	-	-	-	-	5	5	-	-	-	-	-
D	5	5	5	5	10	10	5	5	5	5	5
E	-	-	-	-	-	-	-	-	-	5	5
F	1	1	1	2	1	1	1	1	1	1	1
G	1	1	1	1	1	1	1	1	1	1	1
H	0,05	0,01	-	-	-	-	-	-	-	-	-
I	0,1	0,02	-	-	-	-	-	-	-	-	-
J	-	-	-	-	-	5	-	-	-	-	-
K	-	-	-	-	-	-	2	-	2	2	2
L	-	-	-	-	-	-	-	0,1	-	-	-
M	-	-	-	-	-	-	-	-	1	1	-

A. Dried sugar cane juice "rapadura" (g/l)
B. Yeast extract (g/l)
C. Beef extract (g/l)
D. Peptone (g/l)
E. Tryptone (g/l)
F. Dipotassium phosphate (g/l)
G. Ammonium sulphate (g/l)
H. Manganese sulphate (g/l)
I. Magnesium sulphate (g/l)
J. Sodium acetate (g/l)
K. Ammonium citrate (g/l)
L. Complex B vitamins (Roche) (% v/v of the following solution: thiamine mononitrate 0.5 g/l, Riboflavine 0.2 g/l, pyridoxine hydrochloride 0.2 g/l, nicotinamide 2 g/l, calcium pantotenate 3 (g/l)
M. Tween 80 (g/l)

incubated at 30°C under rotatory shaking (100 rpm). Bacterial growth was measured by absorbance readings every 15 minutes at 600 nm in a Baush and Lomb, Spectronic 20. The growth parameters μ (specific growth rate) and R (lag factor) were determined graphycally, by plotting logarithm of absorbance versus time in computer with an energraphics program.

The initial viable cell number was near 1 x 10^7 /ml. Viable cell number was measured according to the method described by Busta *et al.* (4). The compositions of media tested are shown in Table 1.

The cultivation of microbes in CANA 11 medium at various concentration of CTAB was similar to the method of media selection. The stock solution of CTAB was sterilized by filtration through a membrane filter (Millipore, filter type HA, 0,45 μm pore size) and added to CANA 11 medium prior to culture inoculation.

RESULTS AND DISCUSSION

Two factors were considered for the selection of test medium: (1) ability to promote fast growth of microorganisms after a rather short lag phase and (2) a composition as close as possible to fresh sugar cane juice, since the method is intended for application in sugar cane industry.

"Rapadura", a sweet cake commonly marketed in certain Brazilian regions, is obtained by concentration by heating cane juice up to a point when it becomes solid after cooling. Being stable at room temperature, it has the basic composition of sugar cane juice, is quite soluble, easily filterable if suspended particles need to be removed, and was therefore selected as base for media formulation. In this study, a single batch of "rapadura" was specially made in our laboratory and used throughout the experiments. Products such as molasses, HTM (high test molasses) and cane juice from industry were avoided because they may contain residues of chemicals added during several operations and vary in composition from batch to batch.

Results of growth of various bacteria in different media formulations are shown in Table 2 and the growth curves with media CANA 1 and CANA 11 are illustrated in Figure 1. The microorganisms tested were selected by their frequent appearance in the sugar cane industry and by their nutritional requirements. Species of *Pseudomonas, Bacillus*, enterobacteriaceae (except *Klebsiella terrigena*) and yeasts were not tested because they exhibit vigorous growth in cane juice with no added nutrients. Only *Klebsiella terrigena* was tested as representative of these groups. Test formulations were compared to media typically used for isolation and growth of each group of microorganisms, since the proposed media were expected to give growth parameters values similar to those obtained with routine culture media.

Only *Klebsiella terrigena* was capable of growth in CANA 1 medium (Fig. 1). The amount of magnesium sulphate and manganese sulphate was the same to MRS medium. Magnesium is known to be essential for growth and its deficiency causes long lag and lower viability of cells. Conversely, its excess causes slower growth (15). This behavior may be explained by its role on phosphatase activity, where magnesium acts as an activator at low concentration and an inhibitor at high concentration.

With the reduction of magnesium and manganese salts to one fifth (CANA 2), the

TABLE 2 - Growth parameters of *Leuconostoc mesenteroides, Lactobacillus fermentum, Klebsiella terrigena* in MRS medium, *Acetobacter aceti.* and *A. pasteurianus,* in ACE medium compared to various media formulations with sugar cane juice. Temperature of incubation was 30°C.

	L. mesenteroides		*L. fermentum*		*Kl. terrigena*		*A. aceti*		*A. pasteurianus*	
Media	m (h^{-1})	R**	m (h^{-1})	R	m (h^{-1})	R	m (h^{-1})	R	m (h^{-1})	R
M	0.72	1.0	0.51	1.0	0.85	1.0	0.63	1.0	0.35	1.0
CANA 1	-***	-	-	-	1.1	1.0	-	-	-	-
M	0.73	0.76	0.41	0.93	0.39	1.0	0.57	0.54	0.46	0.70
CANA 2	0.13	0.81	0.24	0.85	0.69	1.0	0.73	0	0.36	0.64
M	0.82	0.87	0.44	1.0	0.55	1.0	0.55	1.0	0.57	1.0
CANA 3	0.37	1.0	0.26	1.0	0.84	1.0	0.48	0.74	0.46	1.0
M	0.79	0.08	0.44	1.0	0.42	1.0	0.50	0.91	0.43	0.37
CANA 4	0.39	0.76	0.57	1.0	0.91	1.0	0.50	0.83	0.51	0.92
M	0.62	0.65	0.34	0.68	0.35	0.86	0.33	0.78	0.17	0.86
CANA 5	0.19	0.74	0.24	0.63	0.60	0.84	0.40	0.44	0.31	0.34
M	0.71	0.72	0.42	0.86	0.36	1.0	0.55	0.78	0.47	0.89
CANA 6	0.34	0.82	0.32	0.84	0.33	1.0	0.07	0.85	-	-
M	0.86	0.63	0.44	0.96	0.82	1.0	0.75	0.46	0.53	0.95
CANA 7	0.42	0.73	0.33	0.95	0.90	1.0	0.38	0.59	0.24	0.98
M	0.69	0.87	0.37	0.73	0.51	1.0	0.70	0.59	0.43	1.0
CANA 8	0.18	0.86	-	-	0.66	1.0	0.42	0.71	0.28	0.95
M	0.87	0.63	0.40	0.88	0.45	0.96	0.52	0.68	0.53	0.78
CANA 9	0.74	0.53	0.41	0.75	0.74	1.0	0.41	0.59	0.34	0.75
M	0.86	0.72	0.36	0.86	0.38	1.0	0.55	0.67	0.41	0.73
CANA 10	0.84	0.31	0.50	0.67	0.66	0.93	0.57	0.69	0.48	0.94
M	0.89	0.75	0.49	0.91	0.51	1.0	0.65	0.93	0.58	1.0
CANA 11	0.85	0.68	0.42	0.92	0.80	1.0	0.56	0.82	0.46	1.0

* μ = specific growth rate (h^{-1})
** R = Lag factor (dimensionless)
*** (-) = no growth

growth of *Lactobacillus* and *Acetobacter pasteurianus* was improved, but their μ and R values were considerably lower than those usually observed in normally used for cultivation. In CANA 3 medium where magnesium and manganese salts were absent, the growth of all microorganisms was improved but maximal populations was very low. For example, *L. fermentum* showed maximum stationary phase (C_M) after 2 hours of incubation. Since the pH effect could have been responsible for this low maximum, the amount of dipotassium phosphate was doubled (CANA 4), but no subsequent improvement was detected. Specific growth rate (μ) of *Lactobacillus fermentum* was higher than that obtained in previous media but R of *Leuconostoc mesenteroides* was lower. Hence, pH was not only responsible for low maximum.

When increasing proteinaceous materials (beef extract and peptone) the growth of *Acetobacter aceti* and *A.pasteurianus* was terminated after 5 and 2.5 h of incubation, respectively (CANA 5). The addition of sodium acetate (CANA 6) improved growth of *Leuconostoc mesenteroides* and *Lactobacillus fermentum*, yet no detectable growth of *Acetobacter* sp. was observed. Replacement of acetate with citrate (CANA 7) improved growth considerably but was not adequate for cultivation of all organisms (Table 2). Replacement of citrate with a pool of B vitamins (CANA 8) reduced growth. Addition of acetate and tween 80 (CANA 9) improved the quality of the medium, but *A. aceti* showed delayed

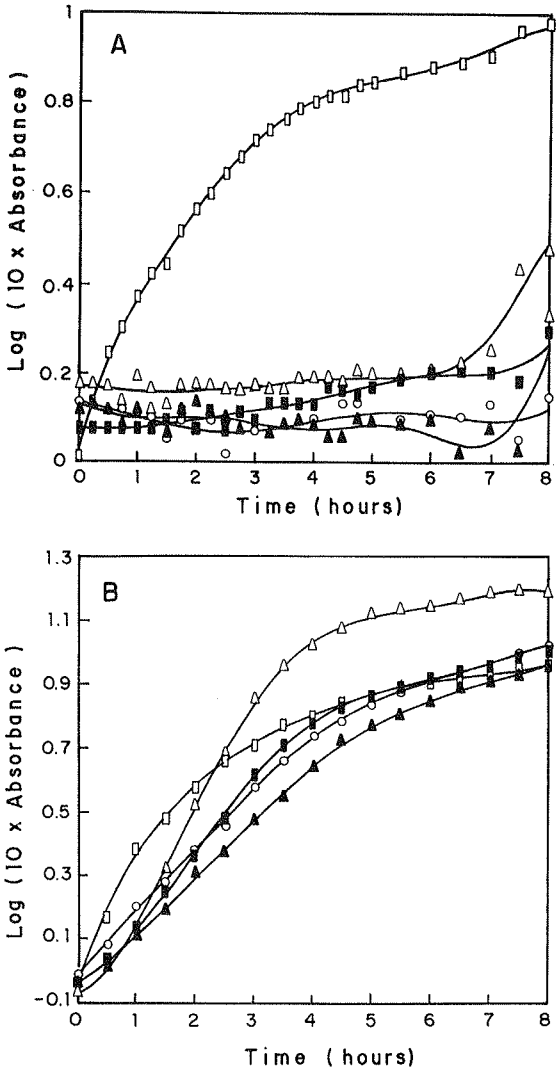

FIGURE 1 - Growth curves of Δ *Leuconostic mesenteroides*, ▲ *Lactobacillus fermentum*, □ *Klebsiella terrigena*, ■ *Acetobacter aceti* and ○ *Acetobacter pasteurianus* in CANA 1 (A) and CANA 11 (B). Temperature of incubation 30°C.

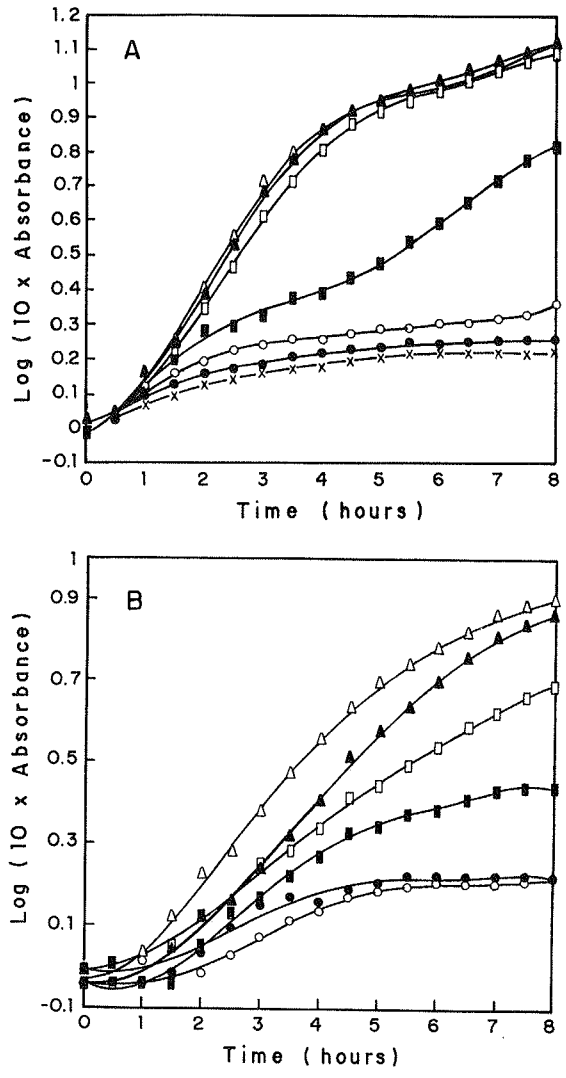

FIGURE 2 - Growth curves of *Acetobacter aceti* (A) in CANA 11 with CTAB, Δ O M, ▲ 0.27 x 10^{-5} M, □ 0.55 x 10^{-5} M, ■ 1.10 x 10^{-5} M and ○ 1.65 x 10^{-5} M, ● 2.19 x 10^{-5} M, X 2.74 x 10^{-5} M and *Lactobacillus fermentum* (B), Δ O M, ▲ 2.74 x 10^{-5} M, ■ 5.49 x 10^{-5} M, ○ 8.23 x 10^{-5} M, ○ 11.0 x 10 M, ● 13.7 x 10^{-5} M. Temperature of incubation 30°C.

growth. Tryptone had a beneficial effect on the growth of all microorganisms (CANA 10), but *L. mesenteroides* showed low R value. When tween 80 was removed (CANA 11) this last problem was overcome (Fig. 1) and this medium was therefore selected for use in all subsequent studies.

EFFECT OF CTAB ON GROWTH PARAMETERS

The effect of various concentrations of CTAB on growth parameters of several microorganisms is shown in Table 3. Some examples of growth curves (*Acetobacter aceti* and *Lactobacillus fermentum*) are illustrated in Fig. 2. Results revealed that CTAB inhibited the growth of bacteria and yeast within a wide range of concentrations. A concentration as low as 5.5 x 10^{-6} M (2 ppm) in CANA 11 medium caused inhibition of *Acetobacter* growth. The most affected growth parameters were specific growth rate (μ) and maximum cell density (C_M), with

negligible effect on lag factor (R). Conversely, *Pseudomonas aeruginosa* and *Pseudomonas cepacia* were highly resistant, presenting only a slight change on growth at 4.1 x 10^{-3} M (1500 ppm).

In general, the microorganisms could be divided into 3 or 4 groups, according to their resistance to CTAB. Group A, corresponding to *A. aceti*, *A. pasteurianus*, *L. fermentum* and *L. mesenteroides* was sensitive to CTAB and showed 50 % inhibition ($\mu=1/2\mu_o$) at a CTAB concentration lower than 1.0 x 10^{-4} M (40 ppm).

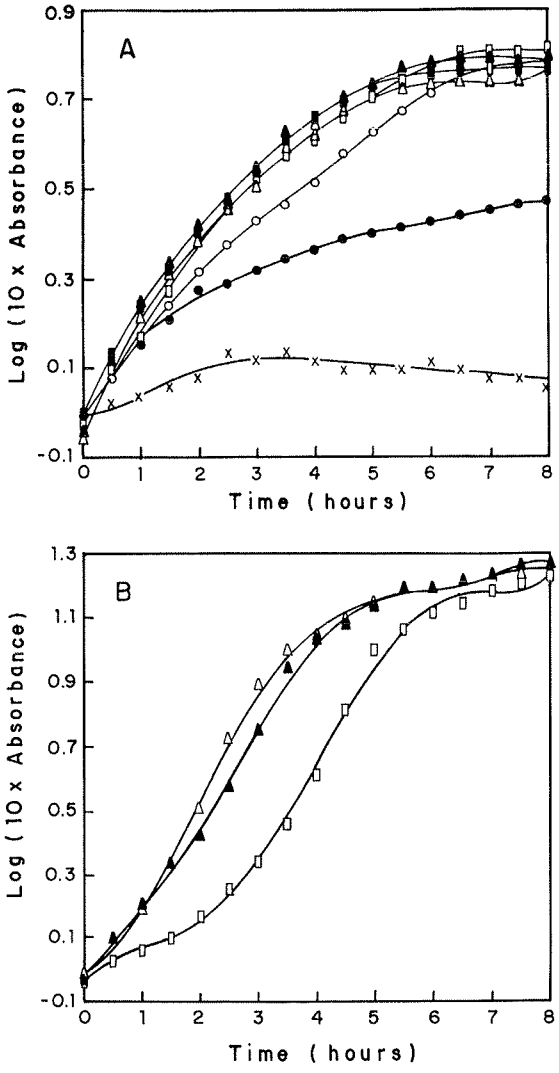

FIGURE 3 - Growth curves of *Escherichia coli* (A) in CANA 11 with CTAB, Δ O M, ▲ 1.37 x 10^{-4} M, □ 2.54 x 10^{-4} M, ■ 4.11 x 10^{-4} M and ○ 5.49 x 10^{-4} M, ● 6.86 x 10^{-4} M, X 8.23 x 10^{-4} M; Temperature of incubation 37°C and *Pseudomonas mendocina* (B), Δ O M, ▲ 5.49 x 10^{-4} M, ■ 5.49 x 10^{-4} M, □ 11.0 x 10^{-4} M. Temperature of incubation 30°C.

Group B, moderately resistant, was represented by *S. cerevisiae* (a yeast) and *Ps. acidovorans*, and was 50 % inhited between 1.0 x 10^{-4} M to 3.0 x 10^{-4} M (40 to 120 ppm). Group C, resistant to CTAB, showed 50 % reduction in μ value within the concentration range of 3.0 x 10^{-4} M to 5.0 or 8.0 x 10^{-4} M (200 to 300 ppm) and was represented by *Escherichia coli* (Fig 3 A), *Klebsiella terrigena* and *Alcaligenes faecalis* in this experiment. The distinction between group B and C was not clearcut and these two groups could be considered as one. Finally, Group D, highly resistant, and consisting by *Ps. aeruginosa*, *Ps. cepacia* and *Ps. mendocina* (Fig.3 B) was not noticeably inhibited by CTAB even at concentration as high as 4.1 x 10^{-3} M (1,500 ppm). It is important to observe that the latter group of microorganisms, μ was not affected but lag factor was lowered at the higher CTAB concentrations. This indicates that some repair or adaptation mechanism of microorganisms was operating at high concentrations of CTAB.

The critical micellar concentration (c.m.c.) of CTAB in CANA 11 medium is close to 1.2 x 10^{-4} M (35), indicating that just highly sensitive bacteria (group A) were affected by CTAB when only monomers of the bactericidal agent were present in the medium. At concentrations of CTAB higher than c.m.c., the following equilibrium system was established:

$$[\text{micelle}] \longleftrightarrow [\text{monomer}]_{\text{solution}} \longleftrightarrow [\text{monomer}]_{\text{cell}}$$

When microbial cells were added, the equilibrium was shifted by removal of monomers thereby changing the c.m.c.

The addition of CTAB to a concentration higher than c.m.c. did not increase monomer concentration, but the inhibition of microorganisms such as *E. coli* and *Kl. terrigena* was higher at greater concentrations of CTAB, indicating that micellar forms also inhibited growth. This inhibition could be caused by the direct action of micelles on the cell envelope or indirectly by complex formation with essencial nutrients such as vitamins or aminoacids in the medium. This contrasts with the germicidal effect of quaternary ammonium compounds measured in deionized water (33).

The results of our experiments showed that there was no relationship between Gram reaction and sensitivity to CTAB. Species of *Acetobacter*

TABLE 3 - Effect of CTAB on specific growth rate (μ) and lag factor (R).

Group A: Sensitive type

CTAB (10^{-5}M)	*L. mesenteroides*		*L. fermentum*		*A. aceti*		*A. pasteurianus*	
	μ (h^{-1})	R	μ (h^{-1})	R	μ (h^{-1})	R	μ (h^{-1})	R
0	0.71	0.54	0.43	0.67	0.61	0.77	0.62	1.0
0.27					0.63	0.69	0.58	1.0
0.55					0.58	0.72	0.55	1.0
1.10					0.33	1.0	0.51	1.0
1.65					0.28	1.0	0.47	1.0
2.19					0.25	1.0	0.43	1.0
2.74	0.63	0.30	0.36	0.65				
5.49	0.43	0.17	0.27	0.78				
8.23	0.21	0.58	0.25	0.77				
11.0	0.13	0.83	0.19	0.73				
13.7	0.12	0.83	0.18	0.77				

Group B: Moderately resistant type

CTAB (10^{-4}M)	*S. cerevisae FPTP 0472*		*S. cerevisae CBS 1171*		*Ps. acidovorans*	
	m (h^{-1})	R	m (h^{-1})	R	m (h^{-1})	R
0	0.47	1.0	0.32	0.85	0.54	0.77
0.55	0.40	1.0	0.32	0.86		
1.10	0.37	1.0	0.33	1.0		
1.37					0.53	0.92
1.65	0.36	1.0	0.30	0.93		
2.19	0.36	1.0	0.24	-***		
2.74	0.32	0.79	-	-	0.11	0.80

Group C: Resistant type

CTAB (10^{-4}M)	*E. coli*		*Kl. terrigena*		*Al. faecalis*	
	m (h^{-1})	R	m (h^{-1})	R	m (h^{-1})	R
0	0.63	1.0	0.60	1.0	0.47	0.97
1.37	0.59	1.0	0.56	1.0		
2.74	0.38	1.0	0.49	1.0	0.48	1.0
4.11	0.38	1.0	0.24	1.0	0.41	0.84
5.49	0.38	1.0	-	-	0.38	1.0
6.86	0.38	1.0	-	-	0.32	1.0
8.23	0.11	1.0	-	-	0.29	1.0
9.63					0.28	0.88

Group D: Extremely resistant

CTAB (10^{-4}M)	*Ps. aeroginosa*		*Ps. cepacia*		*Ps. mendocina*	
	μ (h^{-1})	R	μ (h^{-1})	R	m (h^{-1})	R
0	1.05	1.0	0.60	0.66	0.89	0.90
5.49					0.74	0.65
11.0					0.72	0.27
13.7	0.76	0.97	0.65	0.67		
41.1	0.82	0.77	0.66	0.59		

* μ = specific growth rate (h^{-1})
** R = Lag factor (dimensionless)
*** (-) = no growth

were extremely sensitive and some *Pseudomonas* were extremely resistant, both belonging to Gram negative species. *Lactobacillus* and *Leuconostoc* showed slightly higher resistance than *Acetobacter* were less resistant than *Saccharomyces* species (Table 3). Hence, it is possible to use CTAB to control bacterial contamination in ethanol fermentation process, as long as its concentration is maintained below 2.0 x 10^{-4} M (80 ppm).

The differences in sensitivity might be related to permeability of microbial cells and type of cell metabolism. *Acetobacter* species were extremely sensitive and have only respiratory metabolism. *E. coli* and *Kl. terrigena*, which have both respiratory and fermentative metabolisms, were more resistant. The remarkable resistance shown by *Ps. aeruginosa*, *Ps. cepacia* and *Ps. mendocina* could not be explained by difference in cell metabolism. They grew in the presence of 4.1 x 10^{-3} M (1500 ppm) CTAB with no reduction of growth rate compared to cultures kept in control medium. Some "repair" or "adaptation" process might be involved operating at high concentration of CTAB since these species demonstrated a reduction of R values when 4.1 x 10^{-3} M CTAB was added to growth medium. Such remarkable resistance may explain the cases of bacteremia in hospitals due to the use of contaminated quaternary ammonium solutions (25, 12, 9).

In 1988, Russel and Gould (26) proposed two possible mechanisms of bacterial resistance: intrinsic and acquired forms. Concerning acquired resistance, Townsend *et al.* (34), Tennent *et al.* (31), and Emslie *et al.* (11) reported on resistance of *Staphylococcus aureus* to quaternary ammonium compounds mediated by plasmids. This type of resistance was not found in Gram negative bacteria. The resistance of microbes to hydrophobic and hydrophilic chemicals are examples of intrinsic resistance (19,20,21,24).

Some Gram negative species of bacteria become tolerant to quaternary ammonium compounds by serial transfers to stepwise increasing concentration of the agents (8,6,17,8). *Pseudomonas aeruginosa* resistant to benzalkonium chloride was also shown to be resistant to other quaternary ammonium compounds, yet more sensitive to polymyxin B, colistin sulphate and EDTA (1). *Ps. aeruginosa* resistant to quaternary ammonium compounds produced large amounts of ethyl acetate and ethyl valerate during growth, lost the ability to synthesize extracellular lipases and proteases and was more resistant to osmotic rupture of cells (36).

Additionally, it was also more sensitive to low pH (37), cell size was 30 % smaller than normal and non motile. Besides, the resistant cells showed electron dense bodies of 0.05 to 0.2 μm diameter. *Escherichia coli* resistant to quaternary ammonium compounds also showed higher resistance to sonication (18).

The first suggestion that bacterial lipids may have important role in the resistance of bacterial cells to quaternary ammonium compounds was given by Dyar & Ordal (10). Anderes *et al.* (3), later, reported that *Ps. aeruginosa* grown on medium containing quaternary ammonium compounds had 77% more lipids than sensitive cells. This bacterium grown in medium without agent showed only 27% higher lipids concentration compared to sensitive cells. Lipoprotein content in cell surface of *Serratia marcescens* was higher in resistant type than in the sensitive one (7), and *Enterobacter cloacae* resistant to benzalkonium chloride had more lipids than sensitive strains (22). Resistant *Pseudomonas aeruginosa* had more phospholipids and neutral lipids in cell wall than sensitive cells, and cell permeability was reduced due to higher amount of fatty acids (27).

Many proteins are involved in transport of molecules. These proteins are located in external membrane, amount to approximately a half of the protein mass (24), and form a permeability barrier for many antibiotics (novobiocin, rifamycin, clindamycin) in some Gram-negative bacteria (20).

Therefore, the sensitivity of bacteria and yeast cells to CTAB must be related to difference in metabolisms but the remarkable resistance shown by some species of *Pseudomonas* can only be explained by differences in cell permeability to CTAB. This may be due to special cell wall structure and/or cell membrane compositions. Neutral lipids, phospholipids, lipoproteins and even proteins may be responsible for this property.

Quaternary ammonium compounds, although little effective as germicidal agents due to extreme resistance shown by some species of bacteria, are acceptable as antimicrobial agents in the industrial processing of sugar cane because the relevant microorganisms in this industry are rather sensitive to them. Concentrations of CTAB, as low as 3.0×10^{-5} M (11 ppm) were effective at reducing by 50% the growth rate of *Acetobacter* and lactic acid bacteria in cane juice medium.

RESUMO

Formulação de meio de cultura para cultivo de microrganismos de importância para a industria de açúcar e alcool e emprego de brometo de cetiltrimetilamoneo como agente antimicrobiano

Foi desenvolvido um meio adequado para cultivo de microrganismos importantes na indústria de açúcar e álcool. A eficácia do meio foi determinada medindo-se os parâmetros de crescimento dos principais microrganismos contaminantes do caldo de cana que são exigentes quanto aos fatores de crescimento. Esse meio teve base no caldo de cana desde que a sua finalidade era avaliação dos agentes antimicrobianos comumente usados no controle de contaminação da indústria sucro-alcooleira. Como exemplo de sua utilização, o comportamento do brometo de cetiltrimetilamônio (CTAB) no meio de CANA 11 foi analisado quanto à sua atividade sobre os microrganismos selecionados. Bactérias do ácido acético e do ácido láctico foram os mais susceptíveis seguidas de leveduras, *Pseudomonas acidovorans* e depois pelas enterobactérias. Algumas *Pseudomonas* (*Pseudomonas aeruginosa*, *Ps. cepacia* e *Ps. mendocina*) foram extremamente resistentes ao CTAB, provavelmente em virtude da impermeabilidade do invólucro celular. A eficácia do CTAB em concentrações muito baixas sobre os microrganismos de interesse à indústria sucro-alcooleira pode ser medida por essa técnica.

Palavras-chave: agentes bacteriostáticos, indústria açucareira, brometo de cetiltrimetilamônio.

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EXTRACTION AND EVALUATION OF CRUDE GLUCAN OBTAINED FROM *SACCHAROMYCES CEREVISIAE* CELLS.

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SHORT COMMUNICATION

SUMMARY

Cells of *Sacharomyces cerevisiae* which are largely employed in Brazil for ethanol production were used as raw material for crude glucan isolation. The preparation compares very well with others previously described and was shown to be useful as a thickening agent. The final yield (dry crude glucan/ dried yeast cells) was about 12%.

Key words: yeast cell walls, glucan, food thickening agents

In 1993, Brazilian ethanol distilleries were able to produce more than approximately 250,000 tons per year of dried yeast cells (3,11). Most of them were *Saccharomyces cerevisiae* cells. Although some distilleries are beginning to dry and sell some residual cells, a large amount is still disposed of as useless material (7). Due to the low market value of these yeast cells, we believe that its use as raw material for higher-priced products would be most profitable. Among arious valuable products (yeast extracts, yeast protein isolates, nucleic acid concentrates), a thickening agent (crude glucan) has been successfully obtained from different yeasts (13,14). Crude glucan has been claimed as a very promising agent for food and cosmetic preparations (2,13): it is atoxic and gives a fat-like mouthfeel to low-calory foods. The present note states that a simple extraction method yields a crude glucan preparation very similar to previously described ones. Moreover, this work also demonstrates that the yeast strain most used by the Brazilian ethanol distilleries is an appropriate raw material for crude glucan production.

Baker's yeast (Fleishmann, Brazil) was purchased in local supermarkets as pressed cells (30% dried cells) and used without further purification in all experiments. The extraction method was adapted from previously published data (4,6,9,10) and is presented in Figure 1. The final product was characterized measuring total carbohydrates (8), glycogen (5), glucose and mannose by liquid-gas chromatography as well as crude protein (1) and total fat contents by complete extraction with ethyl ether (12). Mannan was calculated from the mannose content determined by liquid-gas chromatography; the glucan content was computed as total carbohydrates minus the sum of glycogen plus mannan. IR spectrograms were made in KBr pellets (Perkin Elmer model 1720FT-IR) and some data for the rheological characterization were obtained with crude glucan suspensions (LUT Synchro-Letric model, Brookfield Viscometer with a 3C4-31/13 adapter for small samples).

The obtained crude glucan compares well with several similar preparations previously

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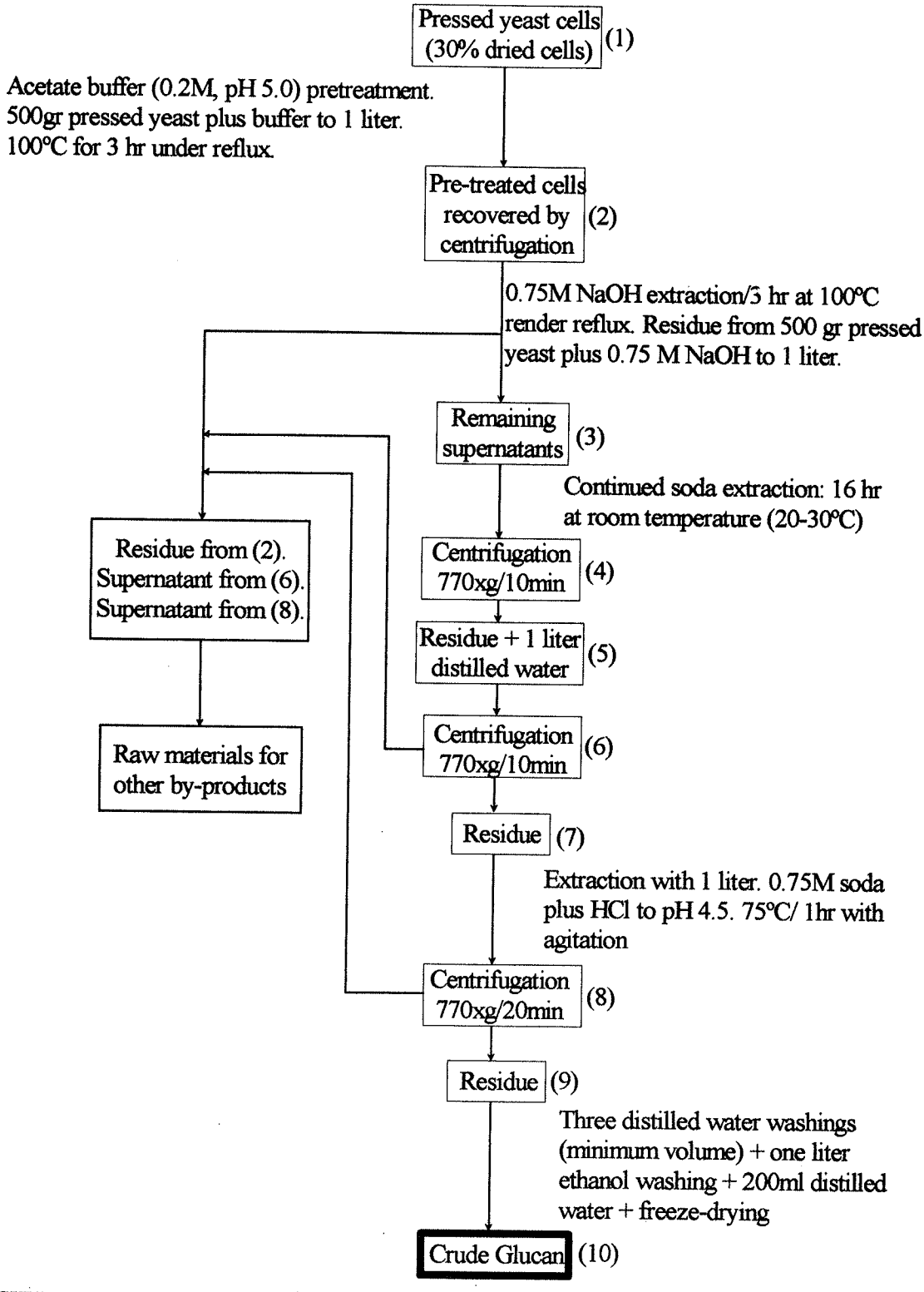

FIGURE 1 - Obtention of a crude glucan preparation from yeast cells by an acid-alkaline procedure.

TABLE 1 - Characteristics of our crude glucan and comparative data from previously described similar prepara tions.

Characteristics	Our preparation	Other preparation
Glucan[1]	48.5 ± 4.4 (n=3)[2]	35.9 - 39.5 (ref. 9)
Total Carbohydrates	91.1 ± 2.5 (n=3)	75.0 - 80.0 (ref. 9)
Glycogen	42.4 ± 2.5 (n=3)	35.6 - 40.5 (ref. 9)
Crude Protein	3.5 ± 1.0 (n=3)	9.8 - 19.5 (ref. 13)
Crude Fat	Ca. 1.8 (n=2)	1.3 - 2.7 (ref. 13)
IR Peaks[3]	7.95,8.31,8.64,9.20 and 11.3 μm (n=2)	7.95,8.31,8.61,9.05 and 11.3 μm (ref. 4)
Yield, %[4]	10.4 - 13.2 (n=2)	9.8 - 15.2 (ref. 9)

1. Total carbohydrates - (Glycogen + Mannan).
2. n = number of replicates.
3. IR spectra of a standard glucan (Laminarin marketed by Sigma Chemical Company) give peaks at 7.95, 8.35, 8.7 and 11.3 μm (14).
4. grams of freeze-dried crude glucan / 100 grams of dried yeast cells

described. Table 1 shows that the main components, the extraction yield and the IR spectrographic characteristics are similar to already published results.

Concerning the rheological characteristics, crude glucan suspensions showed a pseudo-plastic behavior: 7.5% suspensions (w/v) in water and in 0.43M NaCl were measured in shearing rates varying from 0.1 to 100 s^{-1} (25°C) . In all experiments, apparent viscosity decreased with increased shearing rate (0.1 s^{-1} : 1400 - 2000 cP; 100 s^{-1} : below 200 cP). A more complete set of rheological data as well as comments and comparisons with other preparations will be published elsewhere.

Our results suggest that with simple extraction techniques it is possible to obtain a valuable thickening agent from yeast cells used in the Brazilian ethanol industry. Such a fact may constitute a short-term alternative for the convertion of part of the residual cells into a higher-priced product. It should be mentioned that our preparation (as well as other glucan preparations previously characterized) does not possess a thickening power as high as that of many classical thickening agents. In fact, 0.1 - 2.5% pectin or agar-agar or 4-6% gelatin are enough to allow gelation of several food products. When crude glucan was used by Sucher et al. (13) to prepare 05 formulae of food products, reproduction of flavors, mouthfeels, textures and appearance of the respective commercial products were only obtained when using 5.4-10% (w/w) crude glucan concentrations. However, crude glucan alone was able to replace a set of tickening and stabilizing agents in most formulae. Moreover, it is well documented that either post-harvesting treatment of yeast cells or acetic acid extraction of crude glucan do improve the thickening characteristics of the preparations (9,13)

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RESUMO

Extração e avaliação de glucano bruto obtido de células de *Saccharomyces cerevisiae*

Células de uma cepa de *Saccharomyces cerevisiae* muito comuns na produção de álcool no Brasil foram usadas para a obtenção de glucano bruto. O produto tem características muito similares a outras preparações já descritas e demonstrou-se útil como um espessante. O rendimento (glucano bruto seco/células secas de leveduras) foi de cerca de 12%.

Palavras-chave: parede de levedura, glucano, espessante de alimentos.

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ERRATA

Volume **25**(3), 1994

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01 - pg. 137-143 (+ "contents")

Peterkin, P. I.

"New pathogens of interest in food *Vibrio Vulnificus*".

This paper is a REVIEW and **not** a SHORT COMMUNICATION.

The correct title of the article is: "New pathogens of interest in food: *Vibrio vulnificus.*

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02 - pg. 144-148 (+ "contents")

Pereira, M. L.; Buchrieser, C.; Brosch, R.; Catimel, B.; Rocourtz, J.; Hofer, E.

"Characterization of brazilian *Listeria Monocytogenes* strains using DNA macrorestriction patterns"

The correct name of author Rocourtz, J. is **Rocourt, J.**

The correct title of the article is: "Characterization of Brazilian *Listeria monocytogenes* strains using DNA macrorestriction patterns".

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03 - pg. 149-153 (+ "contents")

Asensi, M. D. & Hofer, E.

"Serovars and multiple drug resistant *salmonella* sp. isolated from children in Rio de Janeiro-Brazil"

The correct title of the article is: "Serovars and multiple drug resistant *Salmonella* sp. isolated from children in Rio de Janeiro-Brazil.

The title of the article Portuguese is: Serovares e múltipla resistência a drogas de amostras de *Salmonella* sp. isoladas de crianças no Rio de Janeiro.

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04 - pg. 154-155 (+ "contents")

Saridakis, H. O.

"Non production of Shiga-like toxins by *Escherichia coli* serogroup O_{26}"

The correct title of the article is: Non production of Shiga-like toxins by *Escherichia coli* serogroup O26.

The correct title in Portuguese is: Produção de Toxinas Shiga-like por *Escherichia coli* do sorogrupo O26.

The correct writing of serogroups and serotypes of *Escherichia coli* is O26, O26:H-, O26:H10, O26:H11, O26:H32 and O26:HNT.

The correct writing of SLTs is SLT-I and SLT-II.

---

05 - pg. 161-165 (+ "contents")

Pereira, M. L.; Carmo, L. S. do; Lara, M. A. de; Dias, R. S.; Bergdoll, M. S.

"Enterotoxigenic staphilococci from food handlers working in an industrial kitchen in Belo Horizonte, MG (Brazil)"

The correct address of author Dias, R.S. is: Serviço de Microbiologia de Alimentos; Divisão de Bromatologia e Toxicologia; Fundação Ezequiel Dias - FUNED; Rua Conde Pereira Carneiro, 80; 30510-010; Belo Horizonte - MG - Brasil.

---

06 - pg. 188-194 (+ "contents")

Attili, D. S.; Tauk-Tornisielo, S. M.

"Occurrence of microfungi during leaf litter decomposition in a 'Cerrado sensu stricto' area of São Paulo, Brazil"

The correct name of author Tauk-Tornisielo, S.M. is **Sâmia Maria Tauk-Tornisielo.**

The correct title in Portuguese is "Ocorrência de microfungos durante a decomposição da fração foliar da serrapilheira em uma área de 'cerrado sensu stricto ' de São Paulo.

---

07 - pg. 195-196 (+ "contents")

Moraes-Borba, C. de; Silva, A. M. M. da; Oliveira, P. C. de

"Effect of dithiothreitol and sodium thioglycollate on protoplast production of *Saccharomyces cerevisiae*"

The correct title in Portuguese is: Influência do ditiotreitol e do tioglicolato de sódio na produção de protoplastos de *S. cerevisiae.*

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08 - pg. 197-200 (+ "contents")

Mata, G.; Salmones, D.; Pérez, R.; Guzmán, G.

"Behaviour of some strains of the genus pleurotus after different procedures for freezing in liquid nitrogen"

The correct title of the article is: Behaviour of some strains of the genus *Pleurotus* after different procedures for freezing in liquid nitrogen.

The correct title in Portuguese is: Comportamento de algumas cepas do gênero *Pleurotus* após diferentes métodos de congelamento em nitrogênio líquido.

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