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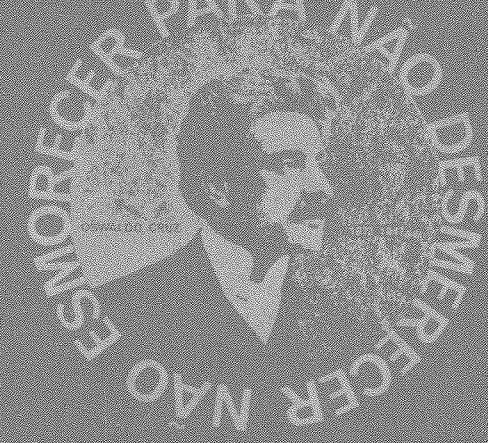

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A quantative study for starvation-induced photoresistance in E. coli

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Summary

Survival curves of *E. coli* K-12 strains, several of them deficient in some DNA — repair process, were obtained after starvation of glucose and growth factors requirements. The curves were mathematically adjusted to a model previously described, which allowed to set up a parameter for expressing the starvation-induced resistence enhancement (SIRE). The experimental results reinforce the presumption that excision repair should be, somehow, involved in SIRE.

Resumo

Estudo quantitativo da fotorresistência induzida por carenciamento em E. coli

Curvas de sobrevivência à radiação ultravioleta de culturas de diversas cepas de *E. coli* K12, proficientes ou não em sistemas de reparação, foram obtidas após carenciamento em glicose e em fatores indispensáveis à multiplicação bacteriana. Estas curvas foram matematicamente ajustadas a modelo previamente descrito, o que permitiu estabelecer parâmetro para expressar o aumento de fotorresistência induzida pelo carenciamento (SIRE). Os resultados obtidos reforçam a hipótese de estar o SIRE relacionado com o mecanismo de reparação por excisão.

Introduction

A mathematical model to fit survival curves (11) was recently modified (17), enlarging its possibilities to describe radiation effects on cells by allowing the definition of several parameters to evaluate the efficiency of some DNA-repair processes (18). According to the alterations proposed, the dependence of survival fraction S on the dose x is expressed by:

$$y = \ell n\, S = Fi(x) + Fr(x) \qquad (I)$$

wherein $Fi(x) = -k_o x$ describes the radiation action on the cell; $Fr(x) = (k_{rec} + k_c)x + Re(x)$ represents the performance of repair systems; $Re(x) = a\,(1 - e^{-bx})$ is to describe particulary the excision repair process (ERP). a was interpreted as being the maximum number of lesions which can be excised and b characterizes the way in which repair saturates with increasing doses. The coefficients k_{rec} and k_c express, respectively, the effectiveness of recombination repair process (RRP) and the enhancement in repair by concomitant action of ERP and RRP (17, 18).

To fit survival curves, the equation I, will be considered in the form:

$$y = \ell n\, S = -kx + a\,(1 - e^{-bx}) \qquad (II)$$

wherein $k = (k_o - k_{rec} - k_c)$ (17).

Based on the modified model, a photoresistance parameter, the mean-survival-dose (MSD), was defined as:

$$MSD = 1/N_o \int_{N_o}^{o} x \cdot dN \qquad (III)$$

wherein dN is the infinitesimal number of cells which becomes inactivated within a range dose between x and $x + dx \cdot N_o$ is the initial number of cells in the culture. The evaluation of the integral, taking into account some simplified conditions (18), leads to:

$$MSD = (1 + a)/k \qquad (IV)$$

In this paper, an expression is proposed in order to evaluate the starvation-induced resistance enhancement (SIRE), based on the MSD-parameter calculated for some *E.coli* strains irradiated after incubation in a liquid medium lacking carbon sources and other essential growth factors such as aminoacids.

Material and Methods

Bacterial strains and media — The thr leu pro his thi arg auxotroph strains *E.coli* AB1157, its derivatives AB1886 (*uvr*A6), AB2463 (*rec*A13), and AB2480 (*uvr*A6 *rec*A13) were described by Howard-Flanders & col. (12, 13). The isogenic thymine auxotroph *E.coli* strains JG113 and JG112 (*polA*1) were described by Gross & Gross (9). Cells were cultivated in BT medium, prepared from the following constituents: Nutrient broth (Difco), 5g; Tryptone broth (Difco), 5g; NaCl (Merck), 5g; distilled water to 1ℓ before autoclavation. Starvation was carried out in M9SS (M9 salts solution) a solution prepared as the M9 growth medium described by Anderson (1) without adding glucose.

Growth, starvation, irradiation and DNA-degradation assays — Log-phase cultures (2×10^8 cells/ml) were prepared from fresh overnight cultures by inoculation of 1 part into 100 parts of BT medium. After filtration through nitrocellulose membranes (Millipore), cells were resuspended in M9SS and divided into two parts: one was immediately irradiated (control) and the other starved for 120 min, at 37°C, with weak aeration. Starvation was carried out in M9SS which was supplemented with thymine (20μg/ml) when thymine-dependent strains were under study, to prevent cell death by thymine deprivation (2, 3, 4). For irradiation, starved cells were filtered and resuspended in M9SS (thymine free). A 25 W-Hg UV-germicidal lamp with almost all the emission at the wavelenght of 253.7nm was used. Bacterial suspensions (6ml) were irradiated at room temperature with gentle stirring in an open 90 by 15mm glass petri-dish. The dose rate was measured by means of a Latarjet meter (14). Manipulations, during and after radiation treatment, were carried out under yellow light. DNA degradation procedures were performed as described elsewhere (23).

Results and Discussion

It has been shown that prestarvation for required aminoacids results in a marked enhancement in ultraviolet resistance for selective *E.coli* strains (2, 3, 5, 8, 23). This UV-resistance enhancement, named SIRE (5), is similarly observed in some *E.coli* cells deprived of carbon sources and growth-factor requirements, as it can be seen in the survival curves presented in Fig. 1. The curves were adjusted to equation II, which coefficients (k, a, b) are presented in Table 1. The SIRE intensity can be expressed as:

$$SIRE\,(\%) = 100\,(MSD_s - MSD_c)/MSD_c \qquad (IV)$$

wherein the s and c indexes refer to starved and control cultures, respectively. The MSD were calculated from the expression IV.

As previously described, some difficulties arise in the interpretation of "broken" exponential curves adjusted to equation II (17, 18), which, sometimes, lead to a negative value for MSD. To overcome this problem for AB2463 strain (Fig. 1B), MSD was determined from the equation of the tangent to survival curve at the origin: $\ell n\ S = -(k + ab)x$ (17). In this case the SIRE value must be considered with some caution.

If SIRE is ascribed to ERP, it would be expected that its intensity would be reduced in *polA*1 mutants.

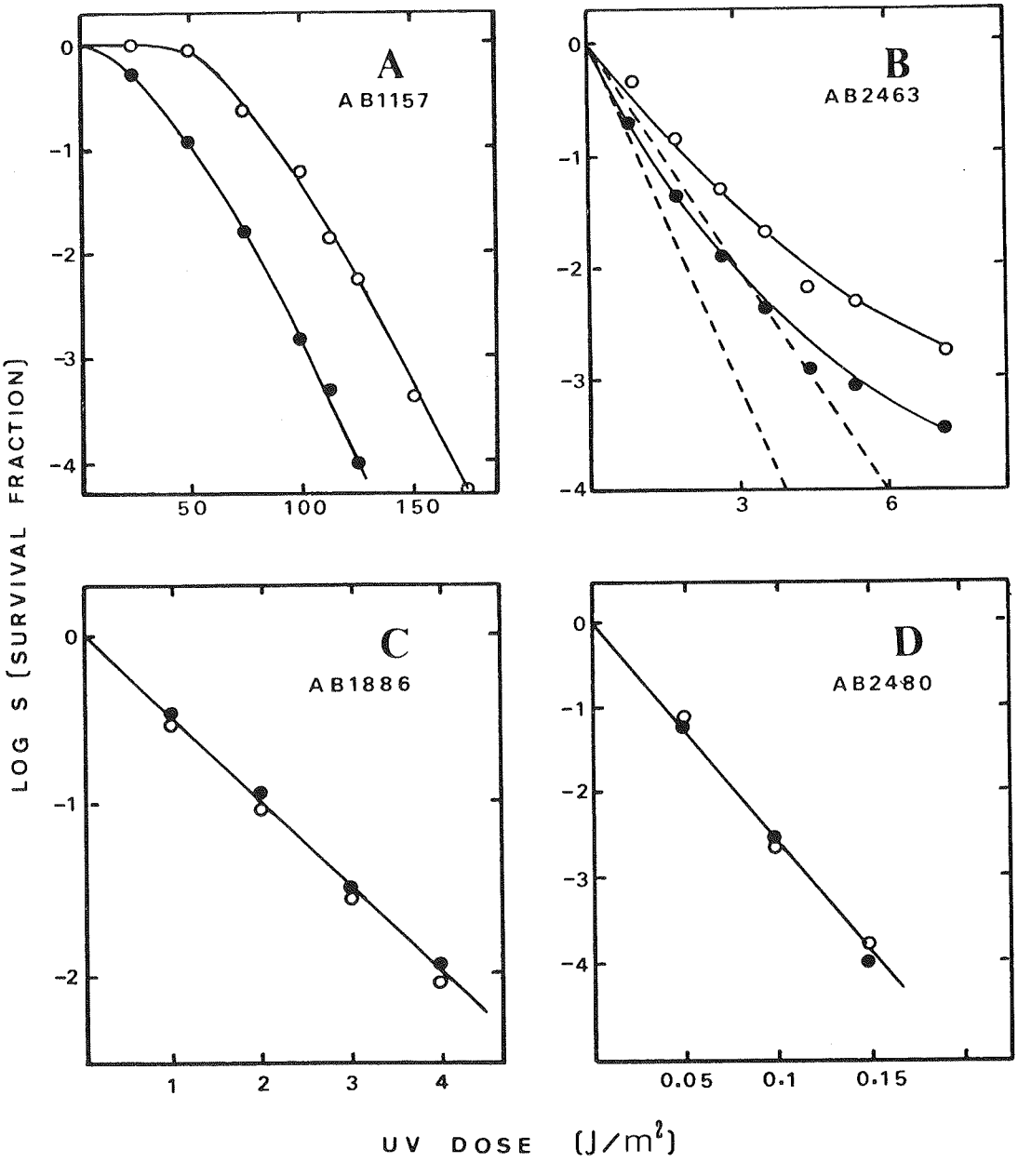
Nevertheless, the SIRE intensity for a polimerase-I deficient strain (Fig. 2B) resulted in 90%, an equivalent value to those (± 85%) found for Uvr$^+$Rec$^+$ cells (Table 1).

Based on DNA-degradation measurements in both starved and log-phase cultures, Masek & col. (15) suggested that prestarvation inhibits the exonuclease action, avoiding large DNA-gaps, which cannot be easily repaired. Similar experiments, carried out with the wild-type and its *polA*1 derivative referred above (data not shown), revealed that the amount of DNA-degradation in log-phase cells was significantly greater than in prestarved cultures, leading to the assumption that starvation could stimulate the excision of large DNA-segments (6) which

would be insoluble in the trichloroacetic acid used in the procedures of measurement. The excision of these large DNA-segments has been made up as an ERP-branch which requires complete growth medium and functional *rec*A, *rec*B, *lex*A, *uvr*D, *pol*C and *lig* genes (24). However, it has been suggested by some authors (7, 26) that this function can be identified with the SOS repair. So, if starvation stimulated this branch of ERP, the SOS repair should be related to SIRE. Nevertheless SIRE is observed in *rec*A mutants as shown by the results presented in Figure 1B.

Experimental evidences indicate that a large amount of dimers remains unexcised in the survivors of aminoacid starved and UV-irradiated

Figure 1 — Effects of growth factors requirements and glucose starvation on survival of *E.coli* K12 strains



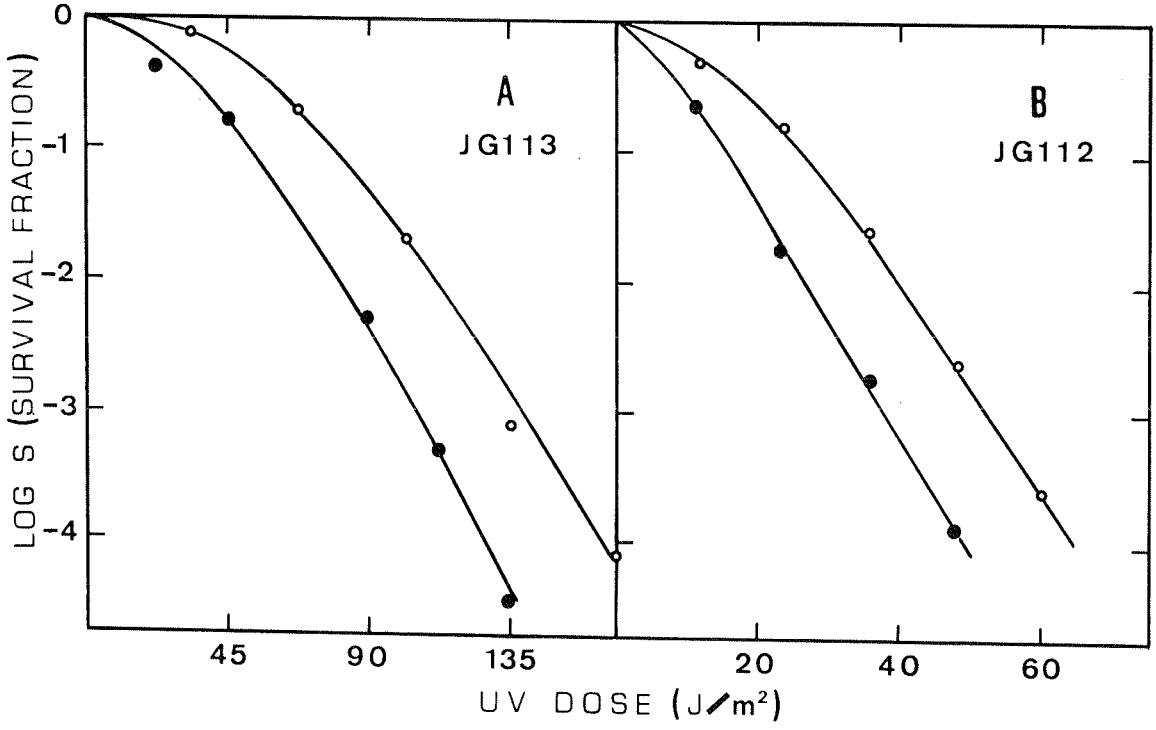
Log-phase cultures of *E.coli* strains AB1157(Uvr⁺Rec⁺), AB2463(*rec*A13), AB1886(*uvr*A6) and AB2480(*uvr*A6*rec*A13) were filtered, resuspended in M9SS, and divided into two parts: one for immediate irradiation (●) and the other for 120 min starvation before radiation treatment (○). Dashed lines (Fig. 1B) are the tangents to survival curves at the origin.

Table 1 — Parameters of UV-survival curve equations obtained for starved and log-phase cultures of *E. coli* K12 strains

Strain	†	k	a	b	MSD	SIRE (%)
AB1157	c	0.115	5.47	0.0210	56.26	82
Uvr⁺Rec⁺	s	0.115	10.82	0.0154	102.78	
AB1886	c	1.15	0	0	0.87	0
(*uvr*A6)	s	1.15	0	0	0.87	
AB2463	c	0.378	−5.80	0.3264	0.43(*)	50
(*rec*A13)	s	0.417	−3.65	0.2994	0.66(*)	
AB2480	c	49	0	0	0.02	0
(*uvr*A6*rec*A13)	s	49	0	0	0.02	
JG113	c	0.110	5.31	0.0210	56.21	85
(PolA⁺)	s	0.110	10.66	0.0120	104.10	
JG112	c	0.21	1.45	0.1493	11.34	90
(*pol*A1)	s	0.18	2.96	0.0719	21.60	

† Simbols: *c* = control (log-phase) culture; *s* = 120 min. starved culture
* These parameters were calculated from the tangent equation of survival curve at the origin: $lnS = -(k + ab)x$ (Fig. 1B).

Figure 2 — Effect of growth factors requirements and glucose starvation on survival of PolA⁺ and PolA⁻ thymine-deficient *E.coli* strains



Cultures of *E.coli* JG113(PolA⁺) and JG112(PolA⁻), were filtered at exponential growth phase, resuspended in M9SS, and divided into two parts: one was immediately irradiated (○); the other was supplemented with thymine (20μg/ml), starved for 120 min., filtered and resuspended in M9SS for irradiation (●).

cultures (19, 21). Studies performed in different excision proficient *E.coli* strains, submitted to treatments which block DNA and protein synthesis prior to UV-irradiation, have shown that, despite a significant depression of dimer excision, a negligible effect on survival was detected. This leads to the consideration that Uvr⁺ cells do not require the elimination of all dimers to survive (20, 21, 22). This assumption can explain the experimental results on DNA-degrada-

tion presented by starved cultures. Taking into account the SIRE intensity obtained for the *pol*A1 mutant, one could consider the hypothesis that starvation activates another ERP-branch, which can be *pol*B or *pol*C dependent (16, 24).

It can be noticed that starvation does not change the *k* parameter values of survival curves (Table 1). Consequently, survival curve asymptotes of starved and log-phase cultures are paralell. The treatment increases *a*, modifying consequently the MSD and SIRE values, mainly for Uvr$^+$ cells. According to experimental data (19, 20, 21, 22) one might believe that SIRE is not exclusively due to remotion of dimers. So, one can postulate that the *Re(x)* function is to describe the cell capacity in excising and tolerating DNA-lesions. The tolerated remaining DNA-defects would be considered as repaired lesions in this mathematical model.

Looking at the *b* parameter (Table 1), which expresses the way in which the mechanisms described by *Re(x)* saturate with increasing doses, it can be noted that its value is smaller for starved than for log-phase cultures, as could be mathematically expected.

It is also known that aminoacid-starved log-phase cells show UV-resistance enhancements which are identical to those observed in stationary-phase cultures (25). This fact has been accounted for as probably due to all starved cells having completed their round of DNA-replication before irradiation. Hanawalt (10) suggested that the reinitiated DNA-synthesis before completion of repair replication in some UV-irradiated log-phase cells does produce DNA-strand breaks (gaps) which are lethal events.

The major part of experimental data induces one to suppose that excision repair is involved in SIRE, despite this phenomenon has been found in Hcr$^-$ strains of *E.coli* B/r (14, 16).

Starvation could also cause a transient cessation of respiration instead of its blockade which would otherwise lead to cell death (10).

Acknowledgements

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Correlação entre soroaglutinação e isolamento de leptospiras em cães

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Resumo

Correlação entre soroaglutinação microscópica e presença de leptospiras nos rins de cães de rua, através de cultura. Observou-se que 16% de cães com sorologia positiva para *canicola* eliminavam este sorotipo de seus rins; 2,8% dos positivos para *icterohaemorrhagiae* eliminavam o sorotipo *copenhageni* e, de 5,5% dos reagentes para *pomona*, foi possível isolar este sorotipo. Dos 35 isolamentos de leptospiras, cinco cepas (14,3%) foram isolados de cães com sorologia negativa.

Summary

The correlation between microscopic agglutination test and the isolation of leptospires in dogs

Correlation between microscopic agglutination test and the presence of leptospires in the kidneys of stray dogs through direct culture. The results indicated that in 16% of dogs with antibodies anti *canicola* this serovar was excreted through the urine; also, in 2,8% with positive serology for *icterohaemorrhagiae* had *copenhageni* in the kidneys and from 5.5% of reagents for *pomona* it was possible the isolation of this serovar. From 35 isolations of leptospires, five (14.3%) strains were recovered from dogs with negative serology.

Introdução

A soroaglutinação microscópica, assim como inúmeras outras técnicas têm sido utilizadas para se determinar índices de infecção e para o diagnóstico das leptospiroses em seres humanos e em animais.

O animal que elimina a leptospira em sua urina, representa um risco para a saúde pública, principalmente quando se trata de animais domésticos.

Em nosso meio, relatam-se freqüentemente inquéritos sorológicos mas, poucas investigações do estado de portador renal de leptospiras têm sido realizadas (4, 7), devido a dificuldades de ordem prática e metodológica, em se proceder a cultura do germe.

Material e Métodos

Foram obtidos 1428 soros de cães de rua, da cidade de São Paulo, durante o período de outubro de 1976 a setembro de 1977. Colheram-se fragmentos de rim, para cultura, de 1415 daqueles cães.

Para o estudo sorológico, empregou-se a técnica da soroaglutinação microscópica, utilizando-se os seguintes sorotipos como antígenos: *icterohaemorrhagiae, canicola, pomona, grippotyphosa, tarassovi, australis, bataviae, ballum, wolffi, panama, pyrogenes, javanica, autumnalis, butembo, andamana, shermani, whitcombi* e *brasiliensis*.

Considerou-se, como soros positivos para leptospirose, aqueles que apresentaram aglutinação igual ou superior a 1:100.

O tecido renal foi colhido com auxílio de pipeta tipo Pasteur, seguindo-se a técnica descrita por Guida (5). O meio de cultura empregado foi o de Fletcher (Difco), enriquecido com 10% de soro de coelho. As culturas foram incubadas a 28°C e observadas em microscopia de campo escuro a cada sete dias, para se detectar crescimento de leptospiras, até o máximo de 50 dias de incubação.

As amostras isoladas foram sorotipadas no Centro Panamericano de Zoonosis, Argentina.

Resultados

Sorologia — Dos 1428 soros analisados, 308 (21,6%) foram reagentes a um ou mais sorotipos. O fenômeno de coaglutinação foi observado em 133 soros. No presente trabalho, para melhor definir o sorotipo infectante nos casos de coaglutinação, seguiu-se a recomendação proposta por Babudieri (3), qual seja, considerar o soro positivo correspondente ao sorotipo de leptospira com o qual se obteve o maior título. Em 38 soros coaglutinantes foram encontrados títulos máximos coincidentes com dois ou mais antígenos e optou-se pela não definição do sorotipo infectante. Assim, em 270 dos 308 soros positivos foi estabelecido o sorotipo responsável pela infecção (Tabela 1).

Cultura — Foram isoladas 35 amostras de leptospiras de 1415 cães (2,5%). O sorotipo *canicola* foi o mais isolado, 32 cepas (91,4%), secundado pelo *copenhageni*, 2 amostras (5,7%) e uma amostra do sorotipo *pomona* (2,8%).

Sorologia × *Isolamento* — A correlação entre os resultados da soroaglutinação e isolamento de leptospiras está representada na Tabela 1.

Nos 38 cães, cujos soros coaglutinaram 2 ou mais sorotipos com títulos máximos coincidentes, obteve-se 5 cepas de leptospiras, todas pertencentes ao sorotipo *canicola*. Nos cães com sorologia negativa isolou-se 5 amostras, todas *canicola*.

Discussão

Menges & col. (8), demonstraram que, após inoculação experimental dos sorotipos *canicola* e *pomona* em 4 cães, era possível reisolá-los 6 dias após. Da urina de dois desses cães obteve-se cultura positiva no 237° dia da inoculação, quando as aglutininas nos soros desses animais já não eram demonstráveis. Os autores enfatizam a limitação do método sorológico, para confirmação da infecção, tanto durante a fase inicial, como no período de portador renal.

Na presente casuística, de 35 cães portadores, 5 (14,3%) não apresentavam aglutininas em seus soros. Tal fato pode ser atribuído tanto a uma infecção no início, quanto ao fato verificado na experimentação acima referida.

Há diferenças no comportamento dos sorotipos *canicola* e *icterohaemorrhagiae*, no que se refere ao estabelecimento do estado de portador renal em cães e conseqüente leptospirúria. Alexander & col. (1), relataram que, nos cães por eles estudados, com sorologia positiva, apenas 10,58% apresentavam leptospirúria. Todos estes animais tinham aglutininas contra *canicola*. Nos cães que apresentaram sorologia positiva para *icterohaemorrhagiae*, não foi observado o mesmo fenômeno. Tais diferenças já haviam sido referidas por Azevedo (2).

O mesmo tipo de comportamento foi observado no presente estudo, onde 16% dos cães, com soros reagentes para *canicola*, apresentaram cultura positiva para o mesmo sorotipo,

Tabela 1 — Resultados da soroaglutinação microscópica, em 1428 soros de cães de rua da cidade de São Paulo e do isolamento de leptospiras em 1415 desses animais, no período de outubro/76 a setembro/77

Sorotipos aglutinados	n.º de soros +	n.º de isolamentos	sorotipos isolados	%
canicola	137	22	*canicola*	16,0
icterohaemorrhagiae	69	2	*copenhageni*	2,8
grippotyphosa	21	0	—	0,0
pomona	18	1	*pomona*	5,5
ballum	12	0	—	0,0
outros*	13	0	—	0,0
Total	270	25		

* outros = *pyrogenes, autumnalis, andamana* e *butembo*.

enquanto apenas 2,8% dos cães, com soros reagentes para o *icterohaemorrhagiae*, mostraram-se portadores, no presente caso, do sorotipo *copenhageni*, que pertence ao grupo *Icterohaemorragia*.

Low & col. (6) inocularam cães com *icterohaemorrhagiae* e verificaram leptospirúria por apenas 2 a 8 dias.

Todas estas observações sugerem uma forte evidência de que *canicola* é o sorotipo melhor adaptado aos cães.

Com relação ao sorotipo *pomona*, parece que este germe se adapta bem aos cães, também. Como referido anteriormente, Menges & col. (8) conseguiram seu reisolamento, 237 dias após a inoculação.

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Pathogenic Escherichia coli associated with infantile diarrhea

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Summary

Of 60 infants younger than one year of age, hospitalized with diarrhea during the summer of 1977-1978 in Recife, 40 (67%) had one or more potential enteropathogenic organisms in their stools. Classic enteropathogenic serotypes of *Escherichia coli* were identified as the sole agent in 11 (18%) patients and associated with other enteropathogens in two cases, as compared with one (5%) of 21 non-diarrheal controls ($P < 0.05$). The most common serotype was 0111:H-. Enterotoxigenic *E. coli* were found in seven (12%) ill children, but none Serény-positive *E. coli* could be recognized. *Shigella* were detected in seven (12%), *Salmonella* in four (7%), and rotavirus in nine (15%) patients. Five (8%) infants were infected with multiple enteropathogens.

Resumo

Escherichia coli patogênica associada à diarréia infantil

Entre 60 crianças, menores de um ano de idade, hospitalizadas com diarréia, durante o verão de 1977-1978 no Recife, 40 (67%) apresentaram um ou mais organismos, potencialmente enteropatogênicos nas fezes. Sorotipos enteropatogênicos clássicos, de *Escherichia coli*, foram identificados como os únicos agentes causais da diarréia, em 11 (18%) pacientes e, associados a outros enteropatógenos, em dois casos, mas em apenas uma (5%), das 21 crianças controle ($P < 0,05$). O sorotipo 0111:H- foi o mais freqüente. Linhagens enterotoxigênicas de *E. coli* foram isoladas de sete (12%) crianças doentes, porém não se detectou qualquer cepa de *E. coli* Serény-positiva. Em sete (12%) pacientes, encontrou-se *Shigella*, em quatro (7%) *Salmonella*, e em nove (15%) rotavirus. Cinco (8%) crianças estavam infectadas com mais de um organismo enteropatogênico.

Introduction

Acute diarrhea has long been a leading cause of infantile death throughout the Third World (12). Therefore, establishment of etiology in diarrhea is strongly desirable if rational control measures have to be taken. In this vein, Guerrant & col. (13) working in the Brazilian city of Florianópolis found that 67% of diarrheal children had enterotoxigenic and/or invasive *Escherichia coli* in their stools. Thinking if this highly impressive finding should be confirmed in other parts of the country, a new investigation has been undertaken in Recife; the results are presented here.

Material and Methods

Patients — Sixty infants under one year of age were selected from a low socio-economic background, with a history of eight or more watery stools in the 24h preceeding admission, and that have not used any antimicrobial drug over the last 30 days (diarrhea group). For comparison, 21 additional infants without diarrhea entering the same hospital units were included (control group). The study was carried out between November 1977 and April 1978; that is during the summer at a room temperature ranging between 21-31°C.

Specimen studies — Stimulating the rectum with a cotton swab one fecal specimen was collected from each child directly into an appropriate vial. Stools were examined promptly for the presence of protozoa (8), and sent immediately to the laboratory for bacteriologic studies. Unprocessed fresh specimens were kept at −70°C until shiped to The Hospital for Sick Children in Toronto, Canada, for rotavirus screening.

Microbiologic methods — For recovering *E.coli* stools were plated directly to MacConkey agar and hektoen agar. Classic enteropathogenic serogroups of *E.coli* (EPEC) were screened in Recife with use of commercial OK antisera (Difco Laboratories, Michigan). Reacting strains were sent to Ida Ørskov at the WHO Collaborative Centre for Reference and Research on Escherichia in Copenhagen for a definitive serotyping. Five to 10 isolates of *E.coli*, after being grown separately, were pooled and screened for toxigenicity and invasiveness. When a positive or an indeterminate result was obtained the individual clones were retested. Heat-labile (LT) enterotoxin production was verified on Y-1 mouse adrenal tumor cells in miniculture (21); 50% or more rounding of cells on two separate assays was considered as a positive reaction. Heat-stable (ST) enterotoxin assay was carried out in the suckling mouse system (1); an average ratio of the weight of intestine of remaining carcasse weight of at least 0.09 was considered as a positive response. Invasiveness was determined by performing the Serény test (23). Furthermore, the isolates of *E.coli* also were screened by slide agglutination against O antisera of known enteroinvasive serogroups (028a,c; 0124; 0136; 0143; 0144; 0152). These strains were kindly supplied by Maria Regina Toledo and Luiz Trabulsi, from São Paulo. Other potential pathogenic enterobacteria were sought and identified by using standard procedures (6). Electron microscopy for detecting rotavirus was done by Peter Middleton, in Toronto.

Results

Infections with EPEC — Pathogenic serotypes of *E.coli* were identified in 13 (22%) of 60 infants with diarrhea, as compared with one (5%) of controls ($P < 0.05$). Two patients presented mixed infections, one with *Shigella* and another with rotavirus. The 14 EPEC strains were distributed among five O groups, and 10 (71%) of them belonged to one of two O serogroups (0111; 0128). The several serotypes of EPEC are listed in Table 1.

Table 1 — Serotypes of enteropathogenic *E. coli* recovered from infants in Recife*

Serotypes	N.º of strains	Characteristic
06:K15:H-	1	LT-ST
09:K84:H21	1	LT
055:H6	1	EPEC
055:H32	1	EPEC
063:H-	1	LT-ST
078:H-:H12	2	ST
089:K-:H-	1	ST
099:K-:H33	1	ST
0111:H-	7	EPEC
0119:H6	1	EPEC
0126:H4	1	EPEC
0128:H-	1	EPEC
0128:H8	1	EPEC
0128:H21	1	EPEC

* Serotyping carried out by Ida Ørskov at the Reference Laboratory in Denmark.
LT = heat-labile toxin
ST = heat-stable toxin
EPEC = classic enteropathogenic serotype of *E. coli*

Infections with toxigenic E.coli (ETEC) — Toxigenic *E.coli* were isolated from sevem (12%) children with diarrhea, as compared with none of 21 controls. One patient was also infected with rotavirus. Among the toxigenic strains, one produced only LT, four produced only ST, and the left two produced both LT and ST enterotoxins. The serotypes of toxin producing isolates also are exposed in Table 1.

Infections with invasive E.coli — Serény-positive *E.coli* were not found at all, despite the fact that 20 lactose-negative isolates of *E.coli* have also been tested for invasiveness. Furthermore, no strain of *E.coli* either lactose-positive or negative were agglutinated with antisera prepared against the so-called invasive serogroups.

Infections with other pathogens — *Shigella* were isolated from seven (12%) children with diarrhea, as compared with none of 21 controls. *Salmonella* were present in the feces of four (7%) diarrheal infants, and in the feces of two (9%) controls. Rotavirus particles were seen in nine (15%) diarrheal stools and in none of controls. *Giardia lamblia* only was seen in one patient, who was also infected with rotavirus.

Discussion

Over the last years, the use of electron microscope and tests for toxigenicity have considerably shorten the number of undiagnosed cases of diarrhea. This was evident in the present survey where a potencial enteropathogen could be recognized in 67% of ill infants. Despite this, EPEC, principally the serogroup 0111, still has been the most frequent organism identified. This experience agrees with previous studies of infant diarrheal disease in Brazil (10), and other parts of the world (2, 17, 20, 22). None of our EPEC isolates were toxigenic nor were Serény-positive. This seems to be a general observation (1, 2, 3, 10, 11, 14, 17) but has brought some doubts on the actual role of EPEC in the pathogenesis of diarrhea, and on whether the routine screening for such organisms would in fact be worth (9, 11). Even though the diarrhea-producing mechanisms of EPEC is yet to be elucidated, recent studies have shown that some of them can produce small amounts of toxin (16), and that they share with *Salmonella* antigens (18), and pili-linked properties (5). In this connection, Reis & col. (19) have found that nine isolates of *E.coli* belonging to serogroup 0128 were able to produce ST enterotoxin. Perhaps, as has been stressed before (7), using of commercial antisera might be responsible, at least in part, for discrepant opinions concerning the value of EPEC as a cause of diarrhea. In present serie, for instance, five isolates of *E.coli* were provisionally misidentified in Recife, but discarded from the enteropathogenic serogroups at the Reference Laboratory in Copenhagen.

Concerning ETEC and invasive *E.coli* the data obtained by Guerrant & col. (13) in Florianópolis could not be confirmed in the present survey. In Recife, the frequency of toxigenic *E.coli* was only 12% and no Serény-positive strains were found at all, in agreement with data observed elsewhere (2, 4, 15). Moreover, recent investigation carried out in São Paulo also detected 12% of toxigenic *E.coli* (Luiz R. Trabulsi; personal communication). Thus, the results observed in Florianópolis must be considered unique and should not be extended to other parts of Brazil.

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Bacteriocins in the genus Azospirillum

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Summary

Twenty four strains of *Azospirillum lipoferum* and 21 of *Azospirillum brasilense* were examined for their ability to produce bacteriocins. Sixteen strains of *A. lipoferum* exerted inhibitory effect on some other strains of this species but not against *A. brasilense* and 3 strains inhibited both species. Some producer strains of *A. lipoferum* were simultaneously sensitive to the bacteriocins produced by other strains of this species. Ten strains of *A. brasilense* inhibited only *A. lipoferum*, two were active against both *A. lipoferum* and *A. brasilense* and one against *A. brasilense* only. None of the producers was sensitive to its own bacteriocins.

Resumo

As bacteriocinas no gênero Azospirillum

Vinte e quatro amostras de *Azospirillum lipoferum* e 21 de *Azospirillum brasilense* foram examinadas em sua capacidade de produzir bacteriocinas. Dezesseis amostras de *A. lipoferum* exerceram efeito inibidor em outras amostras da mesma espécie mas não contra *A. brasilense* e 3 cepas inibiram ambas as espécies. Algumas cepas produtoras de *A. lipoferum* foram simultaneamente sensíveis a bacteriocinas produzidas por outras amostras dessa espécie. Dez amostras de *A. brasilense* inibiram apenas *A. lipoferum*, 2 foram ativas contra tanto *A. lipoferum* como *A. brasilense* e uma contra *A. brasilense* apenas. Nenhum dos produtores foi sensível à sua própria bacteriocina.

Introduction

Isoantagonism produced by related bacterial strains was demonstrated by several authors. Bacteriocins have been found in some genera, but so far not in the genus *Azospirillum*. The concept and the ocurrence of bacteriocins in bacteria have been discussed in some reviews, most recently by Reeves (12). The possible effects of the bacteriocins produced by rhizobia on the formation of the symbiosis between rhizobia and legumes have been studied by Schwinghamer (15) and Schwinghamer & Brockwell (16). The existence of host-plant specifity in the infections of some grasses by *Azospirillum* spp. was observed recently by Döbereiner & Baldani (1). The authors suggested that the differences in sensibility of different strains of *Azospirillum* towards antibiotics produced in host rhizosphere may be one of the factors responsible for the specifity. The ability of a *Azospirillum* strain to produce a bacteriocin-like substance active against other azospirilla could give it an ecological advantage.

This preliminary communication describes the ability to produce bacteriocins in some strains of *Azospirillum* spp. (17).

Material and Methods

Bacterial strains and media — Twenty four strains of *Azospirillum lipoferum* and twenty one strains of *Azospirillum brasilense*, listed in Tables 1 and 2, were examined for interstrain antagonism. All of them came from the collection of Dr. J. Döbereiner (Rio de Janeiro). The examination for antagonism against other genera (the antibiotic activity) was performed using indicator bacteria listed in Table 5.

Table 1 — *Azospirillum lipoferum* strains tested for bacteriocin activity

Strain	Origin
Sp 59b	Wheat roots, Rio de Janeiro
Sp A3a	Grass roots, Dakar, Senegal
Sp USA 5a	Soil under the grasses, Snake River, USA.
Sp RG8b	Wheat roots, Passo Fundo
Sp BR16	Soil under corn, Brasília
Sp BR17	Corn roots, Brasília
Sp RG18b	Wheat roots, Rio Grande do Sul
Sp RG19a	*Digitaria* roots, Rio Grande do Sul
Sp 26HI	Rice roots, Philippines
Sp 108st*	Corn roots, Rio de Janeiro
Sp 113	Corn roots, Rio de Janeiro
Sp 114	Corn roots, Rio de Janeiro
Sp 117	Corn roots, Rio de Janeiro
Sp 121st*	Sorghum roots, Rio de Janeiro
Sp 200	Sorghum roots, Rio de Janeiro
Sp 207	Sorghum roots, Rio de Janeiro
Sp 208	Sorghum roots, Rio de Janeiro
Sp 209	Sorghum roots, Rio de Janeiro
Sp 212	*Panicum* roots, Rio de Janeiro
Sp 214	*Cyperus* roots, Rio de Janeiro
Sp 215	*Brachiaria* roots, Rio de Janeiro
Sp 222	Corn roots, Rio de Janeiro
Sp 229	Corn roots, Rio de Janeiro
Sp 234	Corn roots, Rio de Janeiro

* Streptomycin resistent mutant isolated from potato agar suplemented with 20 $\mu g.ml^{-1}$ streptomycin.

Table 2 — *Azospirillum brasilense* strains tested for bacteriocin activity

Strain	Origin
Sp 7	*Digitaria* roots, Rio de Janeiro
Sp Col 1a	Soil, Columbia
Sp Phl	Rice roots, Philippines
Sp A4	*Panicum* roots, Ibadan, Nigéria
Sp A6	Soil under the grasses, Dakar, Senegal
Sp A7	Rice roots, Ibadan, Nigéria
Sp A8	*Panicum* roots, Ibadan, Nigéria
Sp 13	*Digitaria* roots, Rio de Janeiro
Sp BR14	Wheat roots, Brasília
Sp FL 17	Corn roots, Venezuela
Sp FL 22	*Cynodon dactylon* roots, U.S.A.
Sp 28	*Panicum* roots, Rio de Janeiro
Sp 34	*Digitaria* roots, Rio de Janeiro
Sp 35	*Digitaria* roots, Rio de Janeiro
Sp 67	Corn roots, Rio de Janeiro
Sp 107	Wheat roots, Rio de Janeiro
Sp 107 st nif$^-$	Wheat roots, Rio de Janeiro
Sp 109 st*	Rice roots, Rio de Janeiro
Sp 201	*Brachiaria* roots, Rio de Janeiro
Sp 203	*Panicum* roots, Rio de Janeiro
Sp 204	*Panicum* roots, Rio de Janeiro

* Streptomycin resistent mutant isolated from potato agar suplemented with 20 $\mu g.ml^{-1}$ streptomycin.

The stock cultures of *Azospirillum* were kept on nutrient agar slants (the composition g/l: bacto-peptone-Difco-10; meat extract-Merck — 3; bacto-yeast extract-Difco — 2; NaCl — 5; $MgSO_4 \cdot 7H_2O$ — 0.25; $CaCl_2 \cdot 2H_2O$ — 0.15; $MnSO_4 \cdot H_2O$ — 0.02; bacto-agar-Difco — 15; pH 7.0) or on potato agar slants (2). The stock cultures of other bacteria tested were kept on nutrient agar slants.

Bacteriocin assay — A double layer plate technique was used for bacteriocin assay. The producer strain was transferred from the stock culture to a fresh nutrient agar slant and incubated for 24h at 33°C. The growth was collected, supensed in a small amount of nutrient broth and a loopfull of this thick suspension was spotted on the surface of nutrient agar in a Petri dish. Three producer strains were spotted in duplicate on each plate. After incubation for 48h at 33°C, the plates were exposed to chloroform vapor and then left until the chloroform evaporated.

The same strains which had been tested as producers were also used as indicator strains. The bacteria were transferred from the stock cultures into nutrient agar slants and then into nutrient broth. The cells were incubated at 33°C for 24h and then 0.2ml of the culture was suspended in 3ml of melted soft (0.7%) nutrient agar. The suspension was seeded over the entire surface of agar spotted with the producer strains. After incubation at 33°C for 48h to allow growth of the indicator bacteria, the plates were examined for zones of inhibition. The same technique was used for assays on the spectrum of antibacterial activity against other genera.

Testing for the presence of bacteriophage — Plugs of agar (diameter approximately 3mm) were removed aseptically from inhibition zones produced against sensitive indicator bacteria and placed in 1ml of nutrient broth. Each tube was treated with 0.05ml chloroform. After 30 min. the mixture was centrifugated in a clinical centrifuge for 5 min. and 0.2ml of each supernatant was mixed with 0.2ml of the suspension of the sensitive indicator cells in nutrient broth and 3.0ml of sterile melted soft agar. The mixture was seeded on the surface of sterile nutrient agar in Petri dish. Plates were incubated at 33°C for 24h and then examined for plaques. Two plugs were removed from each inhibition zone and two separate samples were prepared. Each sample was seeded on three replica plates. This experiment was repeated twice. If any plaque appeared, small plug of agar around it was removed and reexamined for the presence of bacteriophage, as described above.

Results

Twenty four strains of *Azospirillum lipoferum* and 21 of *Azospirillum brasilense* were examined for interstrain antagonism. The results are presented in Tables 3 and 4. Nineteen strains of *A. lipoferum* exerted the isoantagonistic activity. Sixteen of them inhibited only the isolates of the same species, and three producers were active also against *A. brasilense*. The producer strains Sp Br16, Sp Br17, Sp RG18b, Sp RG19a, Sp 113, Sp 207, Sp 208, Sp 209, Sp 212, Sp 214, Sp 215 and Sp 234 of *A. lipoferum* were simultaneously sensitive to the bacteriocins produced by some other strains of this species. Among the *A. brasilense* examined, thirteen were bacteriocinogenic. Ten of them inhibited only *A. lipoferum*, two were active against both *A. lipoferum* and *A. brasilense* and one against *A. brasilense* only. None of the producers was sensitive to its own bacteriocins.

The zones of inhibition exemplified on Fig. 1, varied in size, extending from 1mm up to 10mm between the edge of the growth of producer and the growth of sensitive bacteria. The zones were transparent in most cases.

Table 3 — Bacteriocin activity among *Azospirillum lipoferum**

Indicator strains	Producer strains																		
	Sp A3a	Sp BR16	Sp BR17	Sp RG18b	Sp RG19a	Sp 26HI	Sp 108st	Sp 113	Sp 114	Sp 117	Sp 200	Sp 207	Sp 208	Sp 209	Sp 212	Sp 214	Sp 215	Sp 229	Sp 234
A. lipoferum:																			
Sp 59b	–	+10	+5	+10	+9	+5	–	+5	+6	+5	+10	+6	+6	+6	+4	+4	+4	+5	–
Sp RG8b	+2	–	–	–	–	–	–	+1	–	–	–	–	–	–	–	–	–	–	–
Sp BR16	–	–	–	–	–	–	–	+2	–	–	–	+6	–	–	–	–	–	–	–
Sp BR17	+3	–	–	–	–	–	–	–	–	–	–	+5	–	–	–	–	–	–	–
Sp RG18b	+2	–	–	–	–	–	–	–	–	–	–	+5	–	–	–	–	–	–	–
Sp RG19a	–	–	–	–	–	–	+2	–	–	–	+4	+5	–	–	–	–	–	+4	–
Sp 113	–	–	–	–	–	–	–	–	–	–	–	+5	–	–	–	–	–	+6	–
Sp 114	–	–	+2	–	–	–	+2	–	–	–	–	–	–	–	–	–	–	–	–
Sp 121st	–	–	–	–	–	+1	–	+5	+5	–	–	+3	–	–	–	–	–	–	–
Sp 207	–	–	–	–	–	–	+1	–	–	–	–	–	–	–	–	–	–	+5	+5
Sp 208	–	–	–	–	–	–	–	–	–	–	–	+5	–	–	–	–	–	+3	–
Sp 209	–	–	–	–	–	–	–	–	–	–	–	+6	–	–	–	–	–	+7	–
Sp 212	–	–	–	–	–	–	–	–	–	–	–	+5	–	–	–	–	–	+5	–
Sp 214	–	–	–	–			–	–	–	–	–	+5	–	–	–	–	–	–	–
Sp 215	–	–	–	–	–	–	–	–	–	–	–	+4	–	–	–	–	–	–	–
Sp 222	–	–	–	–	–	–	–	–	–	–	–	+3	–	–	–	–	–	+3	–
Sp 234	–	+6	–	+5	–	–	–	–	–	–	–	+6	+10	+10	–	–	–	+5	–
A. brasilense:																			
Sp Pb1	–	–	–	–	–	–	–	–	–	–	–	+6	+3	+3	–	–	–	–	–
Sp A4	–	–	–	–	–	–	–	–	–	–	–	+6	+5	+5	–	–	–	–	–

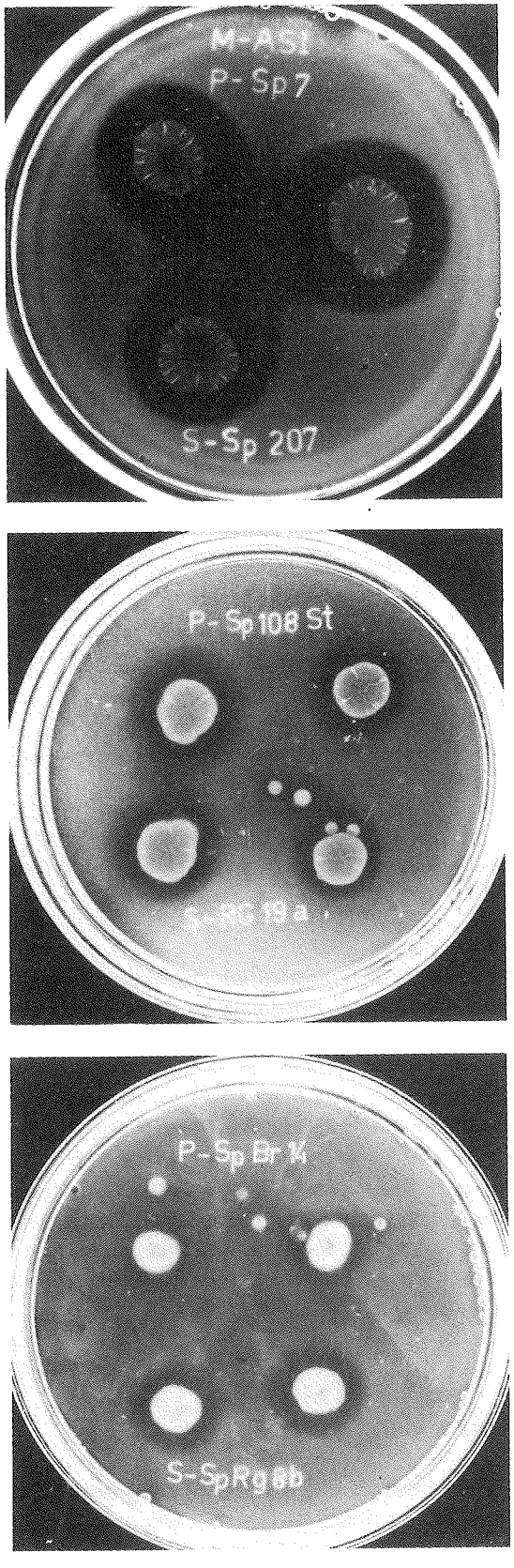
\+ = Clear inhibition zone in lawn of indicator bacteria, in mm
– = No visible zone of inhibition
* = In this table only strains which show positive results (as producer or indicator) are mentioned. For the complete list of azospirilla tested, see Tables 1 and 2.

Table 4 — Bacteriocin activity among *Azospirillum brasilense**

Indicator strains	Producer strains												
	Sp 7	Sp Col 1a	Sp A4	Sp A7	Sp A8	Sp 13	Sp BR14	Sp FL 17	Sp FL 22	Sp 35	Sp 109st	Sp 201	Sp 203
A. lipoferum:													
Sp 59b	+5	+5	+4	+4	+3	+10	(+)3	+10	+10	+5	+2	–	+10
Sp RG8b	–	–	+1	–	–	+1	–	–	–	–	–	–	–
Sp RG19a	–	–	+5	–	–	+5	–	–	+3	+5	–	–	–
Sp 207	+5	–	–	–	–	–	–	+1	–	–	–	–	–
Sp 208		+5	–	–	–	–	–	–	–	–	–	–	–
Sp 234	–	+2	–	–	–	–	–	–	–	–	–	–	–
A. brasilense:													
Sp BR14	–	–	–	–	–	–	–	–	–	–	–	+1	–
Sp 107	–	–	–	–	–	–	–	–	–	–	–	+1	+2
Sp 107st nif⁻	–	+1	–	–	–	–	–	–	–	–	–	+2	–

\+ = Clear inhibition zone in lawn of indicator bacteria, in mm
(+) = Turbid inhibition zone, in mm
– = No visible zone of inhibition
* = In this table only strains which show positive results (as producer or indicator) are mentioned. For the complete list of azospirilla tested, see Tables 1 and 2.

Figure 1 — Inhibition zones in *Azospirillum*



Producer: Sp 7; Indicator: Sp 207
M-ASI = nutrient agar medium

Twenty six strains of non-related bacteria were examined for their sensibility towards the inhibitory factors produced by the bacteriocinogenic strains. The results (Table 5) indicate that ten strains of bacteriocin producing *A. lipoferum* and 1 of *A. brasilense* showed, in addition to the isoantagonistic activity, an antibiotic activity towards some of the non-related bacteria (4 Gram-positives and 6 Gram-negatives).

Thirty-two bacteriocinogenic azospirilla were tested on the phage-like activity (each against only one sensitive strain). The appearance of few small clear inhibition zones similar to plaques caused by phages was noted only in 5 bacteriocin producing strains but not on all replica plates. The attempts to transfer the plaque-forming agent, if any, were unsuccessful.

Discussion

The term bacteriocin is used here to describe an inhibitory agent which causes antagonism between closely related strains. Production of bacteriocins has been observed in various genera of bacteria (5, 12, 13). In this paper, the ability to produce bacteriocin is described for the first time in both species of *Azospirillum* viz., *Azospirillum lipoferum* and *Azospirillum brasilense*.

The inhibition zones considered as positive in our experiments were distinct and resembled those described or shown by other authors (4, 6). However, some of the bacteriocinogenic strains occasionally did not inhibit the sensitive bacteria on all plates in experiments performed in apparently identical conditions, and differences were found even between the activities of replica spots on the same plate (our unpublished data). Similar facts were also noted by other authors (4, 5, 11, 14) in experiments on biological inhibition, performed with other bacteria. In our case the occasional discrepancies may be atributed to the fact that ability to produce bacteriocins is expressed usually by only a small random proportion of cells in the population (10). The proportion of bacteriocinogenic cells may be increased by ultraviolet radiation or by treatment with Mitomycin C (9), but our experiments were performed using uninduced bacteria.

The fact that some producer strains were simultaneously sensitive to the inhibitory factor produced by the strains of the same species has been also observed by other authors (4) and

Table 5 — Antibiotic activity among bacteriocinogenic strains of azospirilla*

Indicator strains	*A. lipoferum* Producer strains										*A. brasilense* Producer strain
	Sp A3a	Sp BR17	Sp RG18b	Sp 26HI	Sp 113	Sp 207	Sp 208	Sp 209	Sp 214	Sp 215	Sp A4
Azotobacter paspali	—	—	—	—	—	—	—	—	—	—	—
Bacillus no. 9	—	—	—	—	—	—	—	—	—	—	—
Bacillus no. 20	—	—	—	—	—	—	—	—	—	—	—
Bacillus megaterium	—	—	—	—	—	—	—	—	—	—	—
Bacillus subtilis	—	—	—	—	—	—	—	—	—	—	—
Beijerinkia camargensi	—	—	—	—	—	—	—	—	—	—	5+
Enterobacter aerogenes	—	—	1(+)	—	—	—	10+	10+	5+	—	—
Enterobacter cloacae no. 1	1(+)	—	—	1(+)	—	—	—	—	—	—	—
Enterobacter cloacae no. 2	—	5+	—		—	5	7+	7+	—	—	—
Escherichia coli	—	—	—	—	—	—	6+	5+	—	—	—
Klebsiella pneumoniae	—	—	—	—	—	—	—	—	—	—	—
Pseudomonas sp. (109M)	—	—	—	—	—	—	—	—	—	—	—
Pseudomonas sp. (1174)	—	—	—	—	—	—	—	—	—	—	—
Rhizobium sp. (J1A)	—	—	—	—	—	—	—	—	—	—	—
Serratia marcescens	—	—	—	—	—	—	—	—	—	—	—
Staphylococcus aureus (ATCC6538)	—	—	—	—	1+	—	—	—	—	—	—
Staphylococcus aureus (Sa-RJ5845)	—	—	—	—	4+	—	—	—	—	—	—
Staphylococcus aureus (Sa-RJ1874)	—	—	—	—	—	—	—	—	—	—	—
Staphylococcus aureus	—	—	3(+)	—	—	—	6+	6+	—	—	—
T 67 (Gram-negative)	—	—	—	—	—	—	—	—	—	—	—
T 73 (Gram-negative)	—	—	—	—	—	—	—	—	—	—	—
T 96 (Gram-negative)	—	—	—	—	—	—	—	—	—	—	—
T 98 (Gram-negative)	—	—	—	—	—	—	—	—	6+	6+	—
E 205 (Gram-negative)	—	—	—	—	—	—	—	—	—	—	—
E 208 (Gram-negative)	—	—	—	—	—	—	—	—	—	—	—
T 314 (Gram-positive)	—	—	10+	—	—	5+	10+	10+	—	2+	—

\+ = Clear inhibition zone in the lawn of indicator bacteria, in mm
(+) = Turbid inhibition zone, in mm
— = No visible zone of inhibition
Strains = T e E — unidentified cerrado soil isolates
* In this table only those bacteriocinogenic azospirilla which show inhibitory activity against non-related bacteria are listed. For the list of all bacteriocinogenic azospirilla, see Tables 3 and 4.

supports our supposition that the isoantagonistic factor found in azospirilla belongs to the class of bacteriocins.

Some of our bacteriocin producing strains were also able to exert antagonistic activity on non-related bacteria. This observation is in accordance with the statements of some other authors (4, 7). The inhibition of growth of non-related bacteria by bacteriocinogenic cells does not imply that the same substances are involved in both bacteriocin and antibiotic activities. The participation of various substances and the existence of various mechanisms in antagonistic activities were documented e.g. in Myxobacterales (3).

The possibility that phages or phage fragments can cause bacteriocin-like effects was suggested by several authors (8, 12). Hallivel & Sweet (4), Jetten & col. (8), McCurdy & MacRae (9) who examined this possibility did not find the direct participation of phages in bacteriocin-like inhibition, in bacteria studied. On the other hand Schwinghamer (15) found that several bacteriocins produced by *Rhizobium trifolii* resembled phages. The participation of defective bacteriophages in bacteriocin-like activity has been also mentioned by Reeves (13). Our occasional observations, performed using the only one technique, suggest the slight possibility of the participation of phage-like activity in isoantagonism among azospirilla. This aspect of bacteriocin action is complex and demands a more thorough examination using the available techniques.

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Characterization of phages of Bacillus subtilis isolated from Brazilian soils

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Summary

Fourteen *Bacillus subtilis* phage isolates, obtained from 5 different soils, were characterized by using plaque morphology, stability at 4°C, electron microscopy, antigenic properties, heat resistance and host range. Use of these criteria permitted their classification in 7 different groups. The largest group consisted of 7 stable, relatively heat resistent and serologically related Bradley type B phages. All other isolates belonged to Bradley type A. The phages are compared to previously described *B. subtilis* phages.

Resumo

Caracterização de fagos de Bacillus subtilis isolados de solos brasileiros

Quatorze fagos de *Bacillus subtilis* foram isolados de 5 solos diferentes e caracterizados quanto a morfologia de suas placas de lise, estabilidade a 4°C, microscopia eletrônica, propriedades antigênicas, resistência ao calor e quanto ao espectro de lise. O uso desses critérios permitiu que estes fagos fossem classificados em 7 grupos diferentes. O grupo maior consiste de 7 fagos estáveis, relativamente resistentes ao calor, sorologicamente relacionados entre si e pertencentes ao tipo B de Bradley. Todos os outros fagos isolados pertencem ao tipo A de Bradley. Os fagos aqui descritos, são comparados com fagos de *B. subtilis* anteriormente descritos.

Introduction

The genus *Bacillus* is a common soil inhabitant (6) and in several Brazilian soils it has been found in quantities up to 10^6 colony forming units (cfu) per gram of dry soil (5). In these samples *Bacillus subtilis* isolates made up a considerable amount (up to 50%) of the total *Bacillus* count (5), which indicates the probable presence of phages associated with this organism.

The primary aim of our research has been to determine the possible role of phages in transduction in soil. To study this, we chose to work with an easily manipulated system, *Bacillus* and its phages. We initially obtained phages from several soils using strains of *Bacillus megaterium*, *B. pumilus* and *B. subtilis* as hosts. The phages of *B. megaterium* and *B. pumilus* will be described elsewhere (In preparation).

In the present report 14 *B. subtilis* phage isolates, obtained from geographically unique habitats, are described. Where possible, these will be compared to previously described *B. subtilis* phages from temperate region soils (1, 3, 8, 14).

Material and Methods

Bacterial strains — Bacillus subtilis strains — LMD 69.3 (168 Wt); LMD 70.64 (Marburg); LMD 47.15 (NRS 231); LMD 48.28 (var. aterrimus); LMD 69.2 (W23 Sm^r); F6; F7.
Bacillus pumilus strains: LMD 48.24 (ATCC 70.61); LMD 68.28 (G6); F10
Bacillus licheniformis: LMD 50.16 (ATCC 40.580)
Bacillus cereus: F12
Bacillus megaterium: F4
Bacillus brevis: F14
Bacillus coagulans: F16
Bacillus species: SW; FS

The strains designated LMD are from the collection of Delft Laboratory of Microbiology.

The F-strains are own isolates from garden soil collected at Fundão island, Rio de Janeiro. Unidentified *Bacillus* strain SW was isolated from polluted estuarine water of Guanabara bay, Rio de Janeiro. Synonym names are shown between brackets.

Soil sampling and sample treatment — Surface soil samples were collected aseptically, passed through a 0.71mm mesh sieve to remove coarse particles and then ground aseptically until powdery.

Isolation of phages — Two own methods, designated A and B, were used. In method A, 5g soil was mixed with 20ml of TY medium (Difco tryptone 10g, Difco yeast extract 5g, NaCl 10g, pH 7.2), supplemented with 2.5×10^{-3}M $CaCl_2$ and 10^{-5}M $MnCl_2$ to enhance phage adsorption (= TYS). After mixing vigorously on a Vortex mixer for 2 minutes, the suspension was shaken at 37°C during 6 hours. After centrifugation (6000xg for 10min.) to remove coarse matter, cells and cellular debris, the supernatant was sterilized by a chloroform treatment (0.2ml/10ml of supernatant). Finally, appropriate dilutions in TY medium were plated out using the double agar-layer (DAL) technique (2) on TYS agar (TYS medium with 1.75% Difco agar) with a strain of *Bacillus* in the top layer (TYS + 0.875% of agar).

Method B consisted of supplementing 5g portions of soil with TYS medium up to 70% of the water holding capacity (W.H.C.) and incubation without shaking at 37°C. At sequential time intervals, 5ml of TY was added to duplicate samples, the mixture was shaken vigorously to suspend phages, then centrifuged to precipitate coarse particles, cells and cellular debris (6000xg, 10min.), and the supernatant was treated with chloroform. Appropriate dilutions (in TY) of the resulting phage suspension were plated out as described before.

Purification of phages — Single-plaque material was repeatedly transferred until plaque morphology remained the same in two sucessive transfers. High titre phage stocks were prepared by infecting cultures of *Bacillus subtilis* F_6 in early log phase at a multiplicity of 0.1-1. After lysis, debris was removed by centrifugation (6000xg for 10min.) and the supernatant was treated with chloroform.

In an alternative method, the top layer of plates showing confluent lysis was removed and shaken in 10ml of TY medium. After removal of debris and agar by centrifugation (10000xg for 10min.), the supernatant was sterilized by treating with chloroform. Phage stocks were stored in screw-capped tubes at 4°C.

Further concentration and purification — To further concentrate phage, poly-ethyleneglycol-6000 (PEG-6000, Riedel de Haen) was added to 100ml lysates at a concentration of 10% (w/v). Phage was allowed to settle overnight at 4°C, and the supernatant was removed by siphoning followed by centrifugation (2500xg for 30min.) of the precipitate with adhering liquid. The phage pellet was resuspended in TMK buffer (0.01 M. Tris, 0.005 M. $MgCl_2$, 0.3 M KCl, pH 7.2), and purified by zone-centrifugation (40,000xg, 90min.) over a sucrose density gradient (5-40%) in TMK. The blueish phage bands were collected and dialysed extensively against TMK. Purified phage stocks were stored at −20°C.

Electron microscopy — Preparations for the electron microscope were made on 200 to 400 mesh formvar-coated copper grids fortified with carbon. These were negatively stained with 2% sodium (dodeca) tungsto silicate (STS) and examined in a Philips EM 301 electron microscope at 80 kV. Calibration of the microscope was done with catalase.

Preparation of antisera — Phage antiserum was prepared by immunization of rabbits. Two rabbits per phage each received 0.5-1ml intravenous injections of purified high titre phage 3 times a week during 5 weeks, and a booster of 3ml in the sixth week. The animals were bled by cardiac puncture 7 days after the last injection. Antiserum was collected and stored in sterile screw-capped vials at −20°C.

Phage neutralization tests — K-values for neutralization were determined as described by Adams (2).

Heat resistance — One ml aliquots of viable phage were plated out to determine their titre, then maintained at 70°C for 10min, immediately cooled on ice, and assayed for remaining viable phage.

Host range determinations — Serial 10-fold dilutions of high titre phage stocks were spotted onto lawns of young bacterial cells. After

drying at room temperature, the plates were incubated at 37°C and scored after 16-24 hours.

Induction of prophage — The technique described by Steensma & col. (13) was used.

Results

Isolation of phages and their occurrence in different soils — Strain *Bacillus subtilis* F_6, which was chosen as the phage host, was isolated from Fundão garden soil. It was a genuine *subtilis* strain, responding in all tests to the description given by Gordon (7). In an initial experiment with a crude enriched soil suspension, this strain supported the growth of 4 phages with different plaque morphology. Isolation techniques A and B enhance the possibility of successful phage isolation since they permit germination and growth of the naturally occurring host populations in the soil. This proved crucial since no phage was isolated without previous enrichment.

Fourteen phages, judged different by their plaque morphology, were obtained from 5 different soils (Table 1). Phages were also observed in Ecologia Soil, but not in Seropédica Soil and Leblon Beach Sand. The phage isolates were named by a letter/number code after the soils they were isolated from and their host (FS_1 is the first *subtilis* phage isolated from Fundão garden soil).

Characterization of phages — Plaque morphology is dependent on plating conditions (11). To minimize variation we maintained constant the age of the host culture, incubation time, and temperature. However, since even slight variations may induce a somewhat different plaque morphology, a one time observation of plaque morphology is not conclusive. Therefore the phages were plated out several times after isolation and the morphology of their plaque was observed. This resulted in an apparent "basic" plaque morphology, on which variations occurred (Table 1). All phage isolates were virulent for their natural host *Bacillus subtilis* and were resistent to chloroform treatment.

Eight isolates maintained their titre for 5 months when stored at 4°C in sterile crude lysate, three phages (FS_6, FS_7, FoS_2) slowly lost titre, and three (FoS_1, IS_1, GS_1) were highly unstable under these conditions. Use of 10% (w/v) dimetlyl sulfoxide (DMSO) and 0.5% glucose with storage at −20°C (17) completely stabilized phages FoS_1 and GS_1 up to 22 days, but only slightly increased the stability of IS_1. Other conservation techniques had a lesser effect (Table 2).

Table 1 — Detection and isolation of *Bacilus subtilis* phages in different soils

Soil*-Texture	Presence of Phages	Isolated Phages	Plaque Morphology**
1. Fundão-Loamy sand	+	FS_1	c/t,r,h
		FS_2	c/t,r,h
		FS_3	c/r,h
		FS_4	c/t,r,h
		FS_5	c,r,h
		FS_6	c,r,h
		FS_7	c,r,h
		FS_8	c,r,h
		FS_9	c,r,h
2. Cerrado-Clay	+	CS_1	c,r,h
3. Forest-Rio de Janeiro	+	FoS_1	t,i,-
		FoS_2	c,r,h
4. Itaguaí-Sand	+	IS_1	c,r,h
5. Gunadu-Clay	+	GS_1	t,p,-
6. Ecologia-Sand	+	—	
7. Seropédica-Sandy Clay	−	—	
8. Leblon Beach-Sand	−	—	

* Soil 2 makes up 25% of the surface of Brazil. Soils 4-7 are typical agricultural soils of the State of Rio de Janeiro, collected at UFRRJ, R.J.

** Only a rough description is given:
c = clear
t = turbid
r = regular round edge
i = irregular round edge
h = presence of halo around core
p = pin-point size.

Table 2 — Stabilization of *Bacillus subtilis* phages after 15 days

Medium*	Temperature (°C)	% of viable phage remaining		
		FoS_1	GS_1	IS_1
TY	4	1.5	0.03	0.01
TY	−20	0.02	13.1	0.03
SCM	4	1.3	0.04	0.03
SCM	−20	1.4	18.1	0.003
SCM + sucrose (20% w/v)	4	12.5	0.09	0.02
SCM + albumin (200μg/ml)	4	1.0	0.04	0.02
SCM + glycerol (20% w/v)	−20	92	23	0.05
DMSO (10% w/v) + glucose (0.5% w/v)	−20	100**	100**	10

* Medium SCM was described by Yehle & Doi (17); DMSO = dimethyl-sulfoxide
** At 22 days of incubation.

Electron microscopy — The 14 phage isolates can be classified using the basic morphological types A and B of Bradley (3). No uncommon morphological features were observed in any of these phages. The type A phages possessed a head with a hexagonal outline and a contractile tail with a base plate of uncertain morphology ("brush-like"). On contraction the base plate became clearer and an unknown number of pins could be observed. From our electron micrographs we could not determine if tail fibres were present in the type A isolates. The type B phages were morphologically similar. They possessed hexagonal heads and long non-contractile flexible tails, without an apparent base plate. In several type B isolates (FS_1, FS_2, FS_3 and FS_9), a single flexible fibre of undetermined length was observed protruding from the tail tip.

Tail lengths and head side-to-side measurements were determined for all phages (Table 3). These combined morphological features show that all 7 type B isolates belong to one group with tails approximately 165nm long and 8.5nm wide and heads of 55nm, possessing 1 tail fibre. Based on morphological data the type A isolates were all judged different (Table 3).

The defective phage PBSY with a head of 45nm and tail of 264nm, was also observed in low amounts in 9 phage lysates (Table 3). This phage was found in old cultures of *Bacillus subtilis* F_6 and it was abundant when the organism was induced by mitomycin C (methods). After induction, a second phage with a long flexible non-contractile tail was observed in quantities substantially lower than PBSY. This phage was morphologically similar to temperate phage SPβ observed in *Bacillus subtilis* 168M (15). Its dimensions (head 75nm; tail 360nm) were similar to those known for SPβ (head 75.6 ± 1.5nm; tail 357 ± 12.2nm).

Table 3 — Characterization of *Bacillus subtilis* phages

Phage	Bradley type	Tail (nm)	Head (nm)	Remarks*	PBSY present	K_{FS_1}	K_{FP_1}	K_{70}	Group
FS_1	B	166	55		+	22	0	3.6	1
FS_2	B	161	57.7		+	10	0	3.8	1
FS_3	B	166	55	f	+	10	0	4.2	1
FS_5	B	167	53		+	11	0	4.3	1
FS_8	B	166	58		+	16	0	4.4	1
FS_9	B	161	57	f	−	7	0	4.4	1
CS_1	B	167	53		−	10	0	4.0	1
FS_4	A	215	91	b	−	6	0	3.5	2
FS_6	A	194	68	b	−	8	0	3.4	2
FS_7	A	197	83	b	−	0	0	1.9	3
FoS_1	A	147	76	b	+	0	0	1.0	4
FoS_2	A	208	83	b	+	0	4	2.9	5
IS_1	A	143.5	91.5	b	+	0	0	1	6
GS_1	A	170	92	b	+	0	0	3.9	7

* f = a single tail fibre protrudes from the tail tip
b = base plate observed.

Antigenic properties — Antisera were obtained for phage FS_1 and a *Bacillus pumilus* phage FP_1 isolated from the same soil and described elsewhere (In preparation). When tested against their antigens, these antisera gave K-values of 20 and 60 respectively, and no cross reactions were observed. Pre-immunization sera did not neutralize any of these phages (K = 0). All 14 phage isolates were tested against the 2 antisera (Table 3). The data show that all 7 Bradley type B isolates were serologically related to FS_1, but not to FP_1, which confirms that they belong to one group. Of the type A phages, only FS_4 and FS_6 were inactivated by FS_1 antiserum. One phage (FoS_2) showed a slight serological relationship (K = 4) with *pumilus* phage FP_1; all other isolates were unrelated to FP_1 (K = 0).

Heat resistance — K-values for heat resistance were calculated from the formula $K_{70} = \log_{10} \frac{\text{titre before}}{\text{titre at } 70°C}$ (9). All phage isolates were fairly heat stable, and none was completely inactivated by the treatment (Table 3). K-values for heat treatment of all type B isolates were consistent (between 3.6 and 4.4), which confirms the relatedness of these phages. Phages FoS_1 and IS_1 were very heat resistent (K = 1). The K-values, obtained for phages FS_4 and FS_6, were similar. These 2 phages both were serologically related to FS_1, but not to FP_1, and belong to the same Bradley morphological type. Although their dimensions, as inferred from electron microscopy, are different, they are judged to belong to a distinct group.

Host ranges — When the phage isolates were tested against 17 strains (described in material and methods) of 7 different *Bacillus* species, only 7 strains showed susceptibility to any of the phages, and 10 were resistent to all phages (Table 4). The host ranges were restricted to strains of the *Bacillus subtilis* genospecies (16): 4 of the lysed strains were *Bacillus subtilis*, 1 was *Bacillus pumilus*, and 2 were unidentified *Bacillus* strains, which had the typical colony morphology of *Bacillus subtilis*. Furthermore, these harboured the defective phage PBSY, which places them in the *Bacillus subtilis/Bacillus pumilus/Bacillus licheniformis* group (13).

Five type B phage isolates lysed these two unidentified strains along with their host, *B. subtilis* F_6. Two phages (FS_2 and FS_5) also were capable of forming plaques on *B. subtilis* F_7. Type A phages FS_4 and FS_6, tentatively grouped together, were different only in relation to strain *B. subtilis* LMD 47.15. Phage isolates FoS_1 and IS_1, which were similar by some criteria (Table 3), show different host ranges and were therefore judged to be different.

Discussion

It is not surprising that *Bacillus subtilis* phages were easily detected in and isolated from most soils, since these soils all had previously shown overall *Bacillus* counts of 10^5-10^6 cfu/g of dry soil (5). However, we cannot explain why no phage was observed in Seropédica Soil. The absence of phage from Leblon Beach Sand is consistent with the low overall *Bacillus* count (10^2 cfu/g of dry soil) of this sample (5).

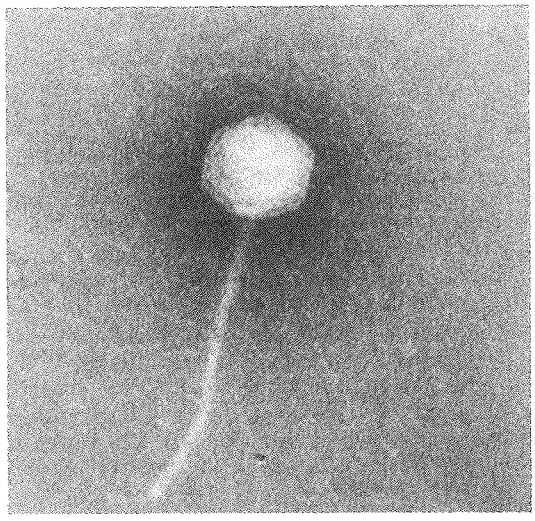
Our hypothesis that the 7 type B phages are members of one group is supported by the data on their plaque morphology, their stability at 4°C, their morphological type (3), the dimensions of their head and tail (Fig. 1), their serological relatedness to FS_1 and their K_{70} value. The observed differences in host range may be due to host range mutations. Moreover, differences are likely to occur between otherwise very similar phage isolates, since in the soil the phages

Table 4 — Host ranges of *Bacillus subtilis* phages*

Bacterial strain	FS_1	FS_2	FS_3	FS_5	FS_8	FS_9	CS_1	FS_4	FS_6	FS_7	FoS_1	FoS_2	IS_1	GS_1
B. subtilis F_6	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis F_7	−	+	−	+	−	−	−	−	−	+	+	−	−	−
B. subtilis LMD 69.2	−	−	−	−	−	−	−	−	−	+	−	−	+	+
B. subtilis LMD 47.15	−	−	−	−	−	−	−	−	+	+	−	−	−	+
B. pumilus 10	−	−	−	−	−	−	−	−	−	−	−	−	+	−
B. spp SW (PBSY)	+	+	+	+	+	+	+	+	+	−	−	−	−	−
B. spp FS (PBSY)	+	+	+	+	+	+	+	+	+	+	+	−	+	+

* Seventeen strains were tested (material and methods). Strains not listed here were not lysed by any of the phage isolates.

Figure 1 — *Bacillus subtilis* phage FP_2. Reference bar indicates 50nm



and their hosts are thought to form a dynamic system in which exchange and modification of genetic material is frequently possible (9). A spectrum of slightly differing phages may exist within a certain basic phage type (defined by morphological features), and results of phage isolations from the same habitat (FS_1 to FS_9) may reflect this. Also, with further characterization it may be possible to group together some of the 16 *pumilus* phages isolated by Reanney (9).

Our isolates FS_4 and FS_6 were tentatively grouped together based on their morphological type, their antigenic relationship to FS_1 and their K_{70} vallue; however, their head dimensions and host ranges were significantly different. The other isolates form 5 distinct groups with 1 member each, since no additional groupings could be inferred from our data.

Data on phage dimensions obtained from different laboratories are not directly comparable, since factors such as different staining techniques, different electron microscopes, and statistic fluctuations when only a small number of phages are measured, may cause differences. Conclusive comparison may only be obtained when the phages are tested in the same laboratory under the same conditions. If the data are viewed this way, a comparison of our phages with literature data showed the following (Table 5):

Table 5 — Comparison between 3 phages

Phage	Bradley type	Tail (nm)	Head (nm)	Fibre (nm)
FS_3	B	166	55	65?*
SF_6	B	165	53	35
SPP_1	B	156	59	30

* The length of the fibre was tentatively determined in one clear picture.

1. Based on the morphological type, dimensions, and the presence of one tail fibre, our type B phages compare to phage SF_6, isolated in the Netherlands from garden soil (12); both our isolates and SF_6 belong to phage species SPP_1, as defined by Ackerman (1). It would be interesting to directly compare these 3 phages.
2. Phage isolate FS_4 (Bradley type A; head 91nm; tail 215nm) could be similar to phage SP-50 (Bradley type A; head 92nm; tail 210nm (4)) which defines the important phage species SP-50 (1). However, we could not determine if a tail fibre was present.
3. The phage isolates FS_1 or FoS_2 (Table 3) may show relationship with TSP_1 (head 90nm; tail 200nm (8)), which also belongs to phage species SP-50 (1). However this phage only forms plaques above 50°C (8), while both FS_7 and FoS_2 are mesophilic isolates.
4. Phage IS_1 (Table 3) shows morphological similary to phage I_9 (head 96nm; tail 145nm; Bradley type A), which was isolated and described by Rima & Steensma (10).
5. Phage GS_1 (Table 3) compares to SP-8 (head 100nm; tail 165nm; Bradley type A (8)). However, Ackerman (1) defined SP-8 as having dimensions of 93 × 152nm. Using their dimensions, phages FS_6 and FoS_1 are not directly relatable to known phages (1, 3, 8, 14), but both are basic type A_1 phages (1) and belong to phage species SP-8 or SP-50.

Future research should further characterize our phages in reference to previously described phages. Neutralization tests with various antisera, determinations of host ranges, burst sizes and other more classical tests as well as characterization of the phage nucleic acid may further elucidate the taxonomic position of these phages.

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Two new leptospiral serovars in the Javanica group isolated in Brazil

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Summary

Two new leptospiral serovars in the *Javanica* serogroup are described. The strains Aa-3 and Aa-4, identical to each other, were isolated from the South American field mouse (*Akodon arviculoides*) in the County of Itaguaí, State of Rio de Janeiro, Brazil. The strain Rr-5 was isolated from a roof rat (*Rattus rattus*) trapped in the same area. The names *fluminense*, strain Aa-3, and *rio*, strain Rr-5, were proposed. The importance of the carrier species is emphasized.

Resumo

Dois novos sorovares de leptospira no grupo Javanica isolados no Brasil

Dois novos sorovares de leptospira no sorogrupo *Javanica* são descritos. As cepas Aa-3 e Aa-4, que são idênticas, foram isoladas do comundongo-do-campo-sulamericano (*Akodon arviculoides*) no município de Itaguaí, Estado do Rio de Janeiro, Brasil. A cepa Rr-5 foi isolada de rato de casa (*Rattus rattus*), capturado na mesma área. Os nomes *fluminense*, cepa Aa-3, e *rio*, cepa Rr-5 foram propostos. A importância das espécies portadoras é enfatizada.

Introduction

Previous observations (1, 2, 3, 4, 7) showed that serveral forms of wildlife, particularly rodents and marsupials, are common leptospiral hosts in Brazil. In addition to known serovars, such as *icterohaemorrhagiae, ballum, australis, pomona, grippotyphosa, wolffi* and *szwajizak*, two strains were isolated and identified as new serovars. They are: 1) *brasiliensis* (8), in the serogroup *Bataviae*, isolated from the opossum (*Didelphis marsupialis*), and 2) *guaratuba* (9), in the serogroup *Pyrogenes*, isolated from the gray "fours eyed" opossum (*Philander opossum*).

In the present study two more new leptospiral serovars are described; they were isolated from the South American field mouse (*Akodon arviculoides*) and the roof rat (*Rattus rattus*). By the agglutinin-aborption test, they are in the *Javanica* serogroup, and are closely related to serovars in this serogroup.

Material and Methods

Two South American field mice (*Akodon arviculoides*) and one roof rat (*Rattus rattus*), trapped live in the field in the District of Seropédica, County of Itaguaí, State of Rio de Janeiro, and registered under numbers 377, 468 and 967 in the "Laboratório de Patologia da Reprodução", were used in attempts to isolate leptospires. Blood and suspensions of macerated kidney tissue from these animals were inocula-

ted into tubes with Fletcher's semisolid medium and intraperitonially into weanling guinea pigs, according to the method of Galton & col. (5). The tubes were incubated at 30°C and examined weekly for growth of leptospires. The temperature of the guinea pigs was measured from the 3rd to the 10th day after inoculation. When anal temperatures of 40°C were seen, the guinea pigs were exsanguinated, and the same materials were used for isolation attempts.

Hyperimmune sera were prepared from the three isolated strains by inoculating live cultures growing in Fletcher's semisolid medium into rabbits weighing about 4Kg.

The leptospiral isolates were adapted to Ellinghausen's liquid medium and presumptively identified by the agglutination screening test with 26 antisera of known serovars, and cross-agglutination studies with live antigens and antisera of serovars of the *Javanica, Icterohaemorrhagiae* and *Celledoni* serogroups. The final typing was done by the cross-agglutinins-absorption test (6).

Results

The cultures were obtained from the kidneys of animal numbers 377 (strain Aa-3) and 967 (strain Rr-5) and from the blood of animal number 468 (strain Aa-4). Leptospires were also recovered from the blood and kidney of the guinea pig inoculated with a suspension of macerated kidney tissue of animal number 468.

The strains Aa-3, Aa-4 and Rr-5 were presumptively classified in the *Javanica* serogroup. The results of the screening test and the cross agglutination studies indicated that these strains were closely related to this group (Tables 1, 2). The strain Rr-5 showed some affinity with serogroups *Icterohaemorrhagiae* and *Celledoni* (Table 3). The subsequent agglutinin-absorption tests (Tables 4 to 8) indicated that the strains Aa-3 and Aa-4, identical to each other, and strain Rr-5 must be considered to be two new serovars in the *Javanica* group. The names *fluminense*, strain Aa-3, and *rio*, strain Rr-5, are proposed.

Table 1 — Agglutination reactions of strains Aa-3, Aa-4 and Rr-5 with antisera against 26 leptospiral serovars of the screening battery

Antiserum		Reciprocal of titer against antigen			
Serovar	Strain	Homologous	Aa-3	Aa-4	Rr-5
ballum	Mus 127	6,400	50	200	—
canicola	Hond Utrecht	25,600	—	—	800
copenhageni	M 20	12,800	—	200	6,400
bataviae	Van Tienen	25,600	—	—	—
grippotyphosa	Moskva V	12,800	—	—	—
pyrogenes	Salinem	12,800	200	200	1,600
autumnalis	Akiyami A	25,600	—	—	—
pomona	Pomona	51,200	—	—	—
wolffi	3705	6,400	—	200	—
australis	Ballico	6,400	—	—	—
tarassovi	Perepelicin	6,400	—	—	—
georgia	LT 117	51,200	—	—	—
javanica	Veldrat B 46	6,400	6,400	6,400	3,200
celledoni	Celledoni	6,400	—	400	400
fort-bragg	Fort Bragg	12,800	—	—	—
sentot	Sentot	3,200	—	—	—
djasiman	Djasiman	25,600	—	—	—
borincana	Hs-622	12,800	—	—	—
cynopteri	3522 C	6,400	—	—	—
butembo	Butembo	12,800	—	—	—
alexi	Hs 616	6,400	—	—	—
panama	CZ 214 K	12,800	—	—	—
shermani	LT 821	12,800	—	—	—
mankarso	Mankarso	6,400	—	—	800
andamana	CH II	51,200	—	—	—
patoc	Patoc I	6,400	—	—	—

(—) negative at 1:50 initial dilution.

Table 2 — Cross-agglutination reactions of leptospiral strains Aa-3, Aa-4 and Rr-5 with members of the *Javanica* serogroup

Antiserum		Titer against antigen				Antisera against antigens in column 1		
Serovar	Strain	Homologous	Aa-3	Aa-4	Rr-5	Aa-3	Aa-4	Rr-5
javanica	Veldrat B46	6,400	12,800	6,400	6,400	1,600	6,400	800
ceylonica	LT 1009	3,200	400	400	1,600	400	—	3,200
poi	Poi	6,400	800	400	200	1,600	200	1,600
sorex-jalna	Sorex Jalná	12,800	200	400	6,400	100	—	50
coxi	Cox	6,400	1,600	1,600	3,200	1,600	—	400
sofia	Sofia 874	6,400	3,200	800	12,800	800	—	1,600
waskurin	63-68	6,400	100	100	51,200	—	—	100
anhoa	90-68	12,800	—	800	6,400	—	—	—

(—) negative at 1:50 initial dilution.

Table 3 — Cross-agglutination reactions of leptospiral strain Rr-5 with members of the serogroups *Icterohaemorrhagiae* and *Celledoni*

Antiserum		Titer against antigen		Rr-5 antisera against antigen in column 1
Serovar	Strain	Homologous	Rr-5	
icterohaemorrhagiae RGA		25,600	1,600	800
copenhageni	M 20	12,800	6,400	200
mankarso	Mankarso	6,400	800	400
naam	Naam	6,400	400	800
mwogolo	Mwogolo	12,800	400	—
dakota	Gran River	25,600	1,600	—
sarmin	Sarmin	6,400	3,200	800
birkini	Birkini	6,400	100	200
smithi	Smith	6,400	200	800
ndambari	Ndambari	12,800	400	—
ndahambukuje	Ndahambukuje	12,800	400	—
budapest	PV-1	25,600	6,400	1,600
weaveri	CZ 380 U	25,600	3,200	6,400
gem	16-67	12,800	800	800
monymusk	75-68	12,800	1,600	800
bog-vere	60-69	12,800	800	50
machiguingia	MMD-3	12,800	6,400	—
celledoni	Celledoni	6,400	400	6,400
whitcombi	whitcomb	6,400	12,800	1,600
new one	52-73	12,800	400	200
new one	27-75	12,800	3,200	6,400

(—) negative at 1:50 initial dilution.

Table 4 — Results of cross-agglutinin-absorption test between the strains Aa-3, Aa-4 and Rr-5

Antiserum Strain	Absorbed with antigen	Reciprocal of titer against antigen			
		Homologous		Absorbing strain	
		Before	After	Before	After
Aa-3	Aa-3	12,800	100	12,800	100
Aa-3	Aa-4	12,800	12,800	200	—
Aa-3	Rr-5	12,800	25,600	800	—
Aa-4	Aa-4	25,600	—	—	—
Aa-4	Aa-3	25,600	—	25,600	—
Aa-4	Rr-5	25,600	25,600	800	—
Rr-5	Rr-5	51,200	100	—	—
Rr-5	Aa-3	51,200	6,400	25,600	400
Rr-5	Aa-4	51,200	6,400	25,600	400

(—) negative at 1:50 initial dilution.

Table 5 — Results of cross-agglutinin-absorption test on leptospiral strain Aa-3 with members of the *Javanica* serogroup

Antiserum	Absorbed with antigen	Reciprocal of titer against antigen			
		Homologous		Absorbing strain	
		Before	After	Before	After
Javanica	Aa-3	6,400	3,200	3,200	—
poi	Aa-3	6,400	12,800	400	—
coxi	Aa-3	12,800	25,600	800	—
sofia	Aa-3	3,200	3,200	400	—
Aa-3	*Javanica*	12,800	25,600	800	—
Aa-3	*poi*	12,800	25,600	3,200	50
Aa-3	*coxi*	12,800	25,600	1,600	—
Aa-3	*sofia*	12,800	12,800	800	—

(—) negative at 1:50 initial dilution.

Table 6 — Results of cross-agglutinin-absorption test on leptospiral strain Aa-4 with members of the *Javanica* serogroup

Antiserum	Absorbed with antigen	Reciprocal of titer against antigen			
		Homologous		Absorbing strain	
		Before	After	Before	After
javanica	Aa-4	6,400	3,200	12,800	—
coxi	Aa-4	3,200	3,200	800	—
sofia	Aa-4	12,800	3,200	800	—
Aa-4	*javanica*	6,400	3,200	1,600	—

(—) negative at 1:50 initial dilution.

Table 7 — Results of cross-agglutinin-absorption test on leptospiral strain Rr-5 with members of the *Javanica* serogroup

Antiserum	Absorbed with antigen	Reciprocal of titer against antigen			
		Homologous		Absorbing strain	
		Before	After	Before	After
javanica	Rr-5	6,400	6,400	800	—
ceylonica	Rr-5	25,600	25,600	1,600	—
sorex-jalna	Rr-5	3,200	1,600	3,200	—
coxi	Rr-5	3,200	3,200	3,200	—
sofia	Rr-5	3,200	1,600	6,400	100
waskurin	Rr-5	51,200	51,200	1,600	—
anhoa	Rr-5	3,200	1,600	1,600	—
52-73	Rr-5	12,800	51,200	400	—
Rr-5	*javanica*	25,600	6,400	800	—
Rr-5	*ceylonica*	25,600	12,800	3,200	—
Rr-5	*sorex-jalna*	25,600	25,600	50	—
Rr-5	*coxi*	25,600	25,600	400	—
Rr-5	*sofia*	25,600	25,600	1,600	100
Rr-5	*poi*	25,600	25,600	1,600	50
Rr-5	*waskurin*	25,600	25,600	100	100
Rr-5	*anhoa*	25,600	25,600	400	50
Rr-5	52-73	25,600	12,800	6,400	50

(—) negative at 1:50 initial dilution.

Table 8 — Results of cross-agglutinins-absorption test on leptospiral strain Rr-5 with members of the *Icterohaemorrhagiae* and *Celledoni* serogroups

Antiserum	Absorbed with antigen	Reciprocal of titer against antigen			
		Homologous		Absorbing strain	
		Before	After	Before	After
icterohaemorrhagiae	Rr-5	25,600	25,600	1,600	—
copenhageni	Rr-5	51,200	51,200	12,800	—
dakota	Rr-5	3,200	1,600	800	—
sarmin	Rr-5	6,400	3,200	12,800	—
budapest	Rr-5	25,600	25,600	6,400	100
weaveri	Rr-5	25,600	25,600	6,400	200
gem	Rr-5	51,200	25,600	1,600	—
monymusk	Rr-5	51,200	51,200	1,600	100
tonkini	Rr-5	3,200	1,600	200	—
machiguingia	Rr-5	6,400	25,600	6,400	400
celledoni	Rr-5	6,400	12,800	800	—
whitcombi	Rr-5	3,200	6,400	12,800	—
27-75	Rr-5	6,400	800	3,200	—
Rr-5	*icterohaemorrhagiae*	25,600	25,600	1,600	—
Rr-5	*dakota*	25,600	12,800	100	—
Rr-5	*sarmin*	51,200	25,600	800	50
Rr-5	*budapest*	25,600	6,400	800	—
Rr-5	*gem*	51,200	25,600	3,200	—
Rr-5	*monymusk*	51,200	12,800	1,600	200
Rr-5	*tonkini*	51,200	51,200	6,400	200
Rr-5	*naam*	51,200	25,600	1,600	100
Rr-5	*smith*	51,200	25,600	1,600	50
Rr-5	*celledoni*	25,600	25,600	6,400	200
Rr-5	*whitcombi*	25,600	25,600	1,600	50

(—) negative at 1:50 initial dilution.

Discussion

According to the results of the cross-agglutinin-absorption tests performed with strains Aa-3, Aa-4 and Rr-5 with members of the serogroup *Javanica*, these strains were new serovars in this serogroup.

The South American field mouse has been shown to be a frequent carrier of leptospires in Brazil. The first leptospiral serovar found in this rodent was *wolffi*, isolated in São Paulo (4). The serovar *grippotyphosa* was isolated several times from the same animal species (7). *Ballum* and *pomona* are two additional serovars recovered from this rodent (2, 9). Since this mouse is widespread over the southeastern region of Brazil, comprising the States of Minas Gerais, São Paulo, Rio de Janeiro and Espírito Santo, it should be considered one of the most important carriers of leptospira in this area.

The roof rat (*Rattus rattus*) is a common commensal found in the Brazilian farms. The serovar *pomona* was isolated previously from this species (2). This was the first time that new serovars were recovered from those species in Brazil.

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Cell wall composition of spores of Hemileia vastatrix (coffee rust)

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Summary

Cell walls isolated from uredospores of *Hemileia vastatrix* contain lipids, polysaccharides, protein, and phosphate. Lipids are the predominant wall components followed by proteins rich in acidic amino acids. Wall polysaccharides contain mannose, glucosamine, galactose, glucose and xylose in a ratio of 8:7:1:0.8:0.2. Glucosamine, liberated from the polysaccharide fraction upon hydrolysis with 6N HCl problably originates from chitin. A mannan was isolated containing equal proportions of 1,3-β and 1,4-β - linked mannopyranose residues.

Resumo

Composição da parede celular de uredosporos de Hemileia vastatrix (ferrugem do café)

A composição química da parede celular de uredosporos de *Hemileia vastatrix* foi analisada verificando-se a presença de polissacarídeos, fosfato, proteína e lipídeos, sendo este último o mais abundante dos componentes analisados. Proteína é o segundo componente em abundância, contendo numerosos resíduos de ácidos aminados acídicos. Manose, glucosamina, galactose, glucose e xilose foram os únicos carboidratos componentes dos polissacarídeos, na proporção 8:7:1:0,8:0,2. A presença da glucosamina foi constatada somente após tratamento da parede com HCl 6N, o que sugere ser proveniente de quitina. A fração de manano isolada continha quantidades equivalentes de resíduos de manopiranose interligados por ligações 1,3-β e 1,4-β.

Introduction

Cell-cell recognition among host cells and pathogenic microorganisms depends ultimately on their surface composition (15). Proteins, glycoproteins and polysaccharides are cell wall molecules which have a sufficiently high structural variability to account for the high degree of specificity of the cell affinity reactions. A further knowledge of the structural components of cell walls may indicate to which extent there is a correlation between cell recognition and phathogenic effects.

Leaves of two cultivars of *Coffea arabica* differing in their susceptibility to race II of *Hemileia vastatrix* (coffee rust) have different cell wall polysaccharides, such differences possibly involving the hemicellulose fraction (11). In the present study we looked at the cell wall composition of *H. vastatrix* in order to understand the plant-fungus association. The chemical composition of the cell walls of natural parasites has been scantily studied in comparison with the numerous data on cell walls from saprophytical yeasts or mycelia obtained in artificial cultures (18).

Material and Methods

Organism — Uredospores of *Hemileia vastatrix* Berk. & Br. were kindly supplied by Dr. Ivan J.A. Ribeiro from the Instituto Agronômico, Campinas, Brazil. The spores belonged to race II which bears the virulence gene v_5 (12) and predominates in Brazil (16). They were obtained from leaves of infected coffee plants grown under asceptic conditions in a greenhouse in Campinas, Brazil. Spore viability was determined by their germination in water. Bacterial contamination was screened as described before (10).

Cell wall isolation, fractionation and analyses — Batches of uncontaminated spores were utilized for the isolation of cell walls following the procedure of Bartnicki-Garcia & Nickerson (1). Cell walls were lyophilized and stored in the freezer under P_2O_5 at −20°C.

The lipid fraction of the wall was obtained by a sequential extraction as described by Bartnicki-Garcia & Nickerson (1). From the lipid-free walls, four other fractions were obtained: soluble in cold KOH, soluble in hot KOH, soluble in hot acetic acid and the insoluble residue. Each of these fractions was analysed for total carbonydrate by the phenolsulphuric acid method (3) as well as for total phosphorus (5), and protein (9).

The intact cell was hydrolysed with 1N and 6N HCl at 100°C for 6h and the resulting reducing sugars, amino sugars, and amino acids were determined by the methods of Nelson (13), Blix (2) and Yemm & Cocking (20) respectively. Hydrolysis products were identified by descending paper chromatography in *n*-butanol-piridine-water (6:4:3 v/v/v) and staining with the alkaline silver nitrate reagent (17). The ratio of sugars in samples was determined by densitometry of spots in the chromatograms using a Canalco Model G densitometer.

For amino acid determination, 10.6mg of walls were hydrolysed with 5ml of 6N HCl in vacuum-sealed vials at 100°C for 18h, and products were analysed using a Beckman model 120C analyser.

Mannan was extracted from intact walls with hot dilute alkali (KOH 2%) and purified by precipitation of the water-insoluble copper complexes formed with Fehling's solution. The purified mannan obtained after de-complexation with 4N acetic acid was hydrolysed with 1N HCl, followed by colorimetry and chromatography as described above. The PMR (proton magnetic resonance) spectrum of the mannan was kindly performed by Dr. P.A.J. Gorin, from the Prairie Regional Laboratory, National Research Council, Saskatoon, Sask, Canada.

Results

The distribution of constituents in the various fractions obtained from the cell walls of *H. vastatrix* is shown in Table 1. Lipid is the predominant component of the cell walls as determined by weighing the extracted fractions.

Phosphate was also found in the lipid fractions, with the bound lipid fraction containing twice as much phosphate as the free lipid fraction.

Protein is the second important cell wall constituent being restricted to the KOH-soluble fractions. Amino acid analysis of cell wall proteins (Table 2) indicated that glutamic and aspartic acids were major amino acids.

Table 2 — Amino acid composition in the acid hydrolysate of the spore walls of *H. vastatrix*

Amino Acid	Per cent of wall weight
Lysine	1.38
Histidine	0.69
Arginine	1.21
Aspartic acid	2.11
Threonine	1.02
Serine	1.42
Glutamic acid	2.29
Proline	0.93
Glycine	1.09
Alanine	1.17
Valine	1.26
Methionine	0.14
Isoleucine	1.19
Leucine	1.78
Total	17.68

Table 1 — Composition of the cell wall from spores of *Hemileia vastatrix* (%)

Fraction	Proteins	Carbohydrate		Phosphate	Lipids	Total
		Neutral	Hexosamine			
1) Free lipid	—	—	—	*	19.0	19.0
2) Bound lipid	—	—	—	*	22.1	22.1
3) 1N KOH, cold	15.6	4.0	—	4.1	—	23.7
4) 1N KOH, 100°C	14.0	2.1	—	4.1	—	20.2
5) 1N acetic acid, hot	0	1.0	—	0	—	1.0
6) Residue	0	2.5	—	0.1	—	2.6
Intact wall (after 6N HCl)	—	—	7.0	—	—	7.0
Total	29.6	9.6	7.0	8.3	41.1	95.6

* The free lipid fraction contains 1.4% phosphate and the bound lipid has 2.4%.

Neutral carbohydrates amount to about 10% of the total wall while hexosamine represents 7%. Chromatography of sugars obtained by acid hydrolysis of walls revealed the presence of mannose, glucosamine, galactose, glucose and xylose (8:7:1:0.8:0.2). The hexosamine content is restricted to the alkali- and acid-insoluble residue (chitin).

Mannan represents approximately 5% of the wall and gave on hydrolysis mannose and traces of glucose. According to the PMR spectrum, this mannan has equivalent numbers of 1,3-β and 1,4-β-linked mannose residues, similar to the mannan isolated from *Rhodotorula glutinis* (6).

Discussion

The cell wall composition of spores of *H. vastatrix* is similar to that of other rusts which have been analysed previously (7, 14, 18, 19). It is noticeable that the lipid fraction represents nearly 50% of the wall, whereas the total carbohydrate fraction less than 20%. A large lipid content which increases the wall hydrophobicity may help uredospore dispersion, prevent dehydration and be a barrier against toxic substances, as suggested for other spores rich in waxy material (4, 8).

The coffee rust mannan is similar to the mannans isolated from uredospores of *Puccinia graminis tritici* (7, 14), *Uromyces phaseoli* var *typica* (18) and *Rhodotorula glutinis* (6). On the basis of mannan structure therefore, specificity of *H. vastatrix* race II to *Coffea arabica* cv. Mundo Novo cannot be explained. Also, the predominance of aspartic and glutamic acids in the *H. vastatrix* spore wall proteins is not a unique feature of this species. Trocha & col. (18) reported a predominance of these acidic amino acids in the uredospore wall of *Uromyces phaseoli*, another rust fungus. Whether the structure of these proteins and/or the structure of the glucose-, xylose-, galactose-containing polysaccharides are those responsible for the specificity of the parasitism of coffee rust by *H. vastatrix* needs further investigation.

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