Rev. Brasil. Genet. 14, 4, 859-871 (1991)
(Brazil. J. Genetics)

GENETIC CHARACTERIZATION OF LACTOSE FERMENTATION AND ANTIBIOTIC RESISTANCE IN PLASMID-HARBORING ENTERIC BACTERIA ISOLATED FROM AMAZON AMPHIBIANS

Luzia Mitie Ioshimoto[1], Lucile Maria Floeter-Winter[2], Carlos Eduardo Winter[2], Willy Beçak[1] and Maria Heloiza T. Affonso[2]

ABSTRACT

Aerobic gram-negative bacteria were isolated from the feces of 20 wild amphibians. The animals were collected from regions flooded by the waters of the Tocantins river during the building of the Tucuruí-PA hydroelectric plant. Of the 420 isolates tested for the presence of plasmids, 123 (29%) showed at least one plasmid DNA band.

Among the 123 plasmid containing isolates, 89% were lactose fermenting, 41% showed resistance to ampicillin, 6.5% to streptomycin and 6.5% to tetracycline, at levels $\geq$ 10 µg/ml.

The most prevalent genera identified among the 123 bacterial isolates were the *Citrobacter* (67%), followed by *Enterobacter* (16%), *Klebsiella* (6%), *Providencia* (3%), *Escherichia* (3%), *Proteus* (2%), *Aeromonas* (2%) and *Salmonella* (1%).

As most of the detected plasmids were large enough to carry genes for conjugal transfer, the relationship between the presence of plasmids and characteristics such as lactose fermentation and drug resistance was analyzed by mating to *E. coli*. The presence of these genes was also examined by DNA-DNA hybridization, using specific probes.

[1] Serviço de Genética, Instituto Butantan, Av. Vital Brasil, 1500, 05504 São Paulo, SP, Brasil. Send correspondence to L.M.I.

[2] Departamento de Parasitologia, Instituto de Ciências Biomédicas, USP, 05508 São Paulo, SP, Brasil.

INTRODUCTION

Data on aerobic fecal flora of amphibians, as well as of other cold blooded animals, are scarce. The reports deal mainly with the occurrence of pathogenic species or bacteria associated with morbidity or high mortality levels of amphibians kept in laboratories (van der Waaig *et al.*, 1974; Carr *et al.*, 1976; Gossling *et al.*, 1982). Wild animals, including reptiles kept in zoos or laboratories have been studied as a potential reservoir of the genera *Salmonella* and *Arizona* (Iveson *et al.*, 1969; Lins, 1971). *Citrobacter* and *Proteus* have been found to be the predominant genera in the feces of several cold blooded animals (Moreno *et al.*, 1973; Roggendorf and Muller, 1976).

There are no references on the incidence or distribution of plasmids in enterobacteria of such animals; the literature has been restricted to papers reporting the presence of plasmids in bacteria of medical importance (Anderson, 1968; Christiansen *et al.*, 1973; Foster, 1983; Edwards and Ewing, 1986) or plant pathogens (Gross *et al.*, 1979). A few ecological studies evaluating the incidence of plasmids in naturally occurring bacteria have been reported (Hada and Sizemore, 1981; Burton *et al.*, 1982; Glassman and MacNicol, 1984; Kobori *et al.*, 1984). The rate of incidence in bacteria isolated from sea water, rivers, ice and soil has ranged from 23 to 42% (Kobori *et al.*, 1984).

In the present study we initially attempted to determine the occurrence and distribution of plasmids in aerobic gram negative bacteria isolated from the bowels of 20 wild amphibians, collected in a pristine area. Afterwards the relationship between the presence of plasmids and some characteristics was analyzed by mating to *E. coli*, curing and DNA-DNA hybridization. The criteria established to select characteristics to be analyzed were based on patterns found in enterobacteria of medical or veterinary importance. Some easily detectable characteristics frequently associated with the presence of plasmids such as drug resistance, lactose fermentation and hemolysin and colonization factor production were investigated. Plasmids with a molecular weight greater than 23 MDa, essential for conjugation (Willets and Wilkins, 1984), were preferentially analyzed through mating.

MATERIALS AND METHODS

Animals

Twenty wild amphibians (Table I) were captured in a region flooded by the waters of the Tocantins river, during the building of the hydroelectric plant of Tucurui (Pará, state-Brasil). The animals belonging to nine species (Table I), were not fed after having been captured in their natural environment. Feces were collected immediately

after the arrival of the animals in the laboratory and streaked onto MacConkey agar medium.

Table I - Identification of plasmid-harboring enteric bacteria isolated from the amphibians.

Animals			Bacteria								
Specie	Code number	Number of animals	*Citrobacter*	*Enterobacter*	*Klebsiella*	*Providencia*	*Proteus*	*Escherichia*	*Aeromonas*	*Salmonella*	Unidentified
Adenomera andreae	1	1	1								1
Bufo typhonius	2	1									
Hyla boans	3	3	23	5	4	2				1	
Hyla hayi	4	2	11	2							
Hyla raniceps	5	1	8	5							
Hyla gr. rubra	6	3	16								
Leptodactylus podicypinus	7	6	20	6				2	1		1
Leptodactylus pustulosus	8	2	1		2		4				
Sphaenorhyncus lacteus	9	1	3		1	2			1		
Total		20	83	18	7	4	4	2	2	1	2

Bacteria

Morphologically different colonies were picked from feces-streaked MacConkey agar plates. A total of 420 colonies from all the animals were purified and analyzed for plasmid content. *E. coli* K12 reference strains C600, 711 and TB1 containing plasmid standards: pUC 19; pBR322; Sa; RP4; VR538 drdl; p307; pF'Lac; R124 and pVC2, which is pBR322 with the lac gene of *Streptococcus lactis* (von Wright *et al.*, 1986), were used.

Plasmid isolation

Bacterial isolates were screened for plasmid content (see Eckhardt, 1978). For other purposes such as plasmid molecular weight evaluation, concentration and purification, the method for preparing clear lysates described by Kado and Liu (1981) was applied. Plasmid size was estimated by comparison with standard plasmid sizes pJPN

(66 Mda), p307 (54 Mda), RP4 (34Mda), Sa (23 Mda), pBR322 (2,8 Mda). Agarose gel electrophoresis was performed in a vertical slab gel apparatus. Gel concentrations ranged from 0.7 to 1.0% in TEB (89 mM Trizma base; 2.4 mM Na_2EDTA and 89 mM boric acid, pH 8.2).

Large-scale isolation of plasmids with CsCl-ethidium bromide gradients was performed as described by Maniatis *et al.* (1982).

Identification of isolates

Plasmid-harboring bacterial isolates were identified by a series of biochemical tests made with a commercially available kit (ENTEROKIT B - PROBAC DO BRASIL, São Paulo, Brasil) and also by additional sugar fermentation tests (Edwards and Ewing, 1986).

The bacteria were coded by two letters: the first one (in upper-case) indicating the genus, and the second the species. The letters were followed by two numbers: the first corresponded to the amphibian code number, and the second to the number of the colony, within each genus, eg. Cf 3.01, means: *Citrobacter freundii* (Cf), isolated from *Hyla boans* (code number 3), the first isolate identified as *Citrobacter* (1).

Antibiotic resistance test

Plasmid-harboring isolates were analyzed for resistance to six antibiotics, ampicillin (Ap), kanamycin (Km), chloramphenicol (Cm), streptomycin (Sm), tetracycline (Tc) and nalidixic acid (Nal) by the method of serial dilutions (Zahner and Maas, 1972).

Hemolysin production

Strains cultured overnight were spotted onto sheep-blood agar plates and observed for the formation of a clear lysis zone around the colonies after 18-19 hours of incubation at 37°C.

Mating experiments

Isolates with resistance to the antibiotics at 10 μg/ml or above this level, as well as lactose fermenting plasmid-carrying strains were mated to *E. coli* K12 C600 (lac^-, thr, leu, thy, thi, Nal^r) according to Affonso *et al.* (1977). MacConkey agar supplemented with Ap, Sm or Tc and Nal for antibiotic resistant isolates and minimal media supplemented with lactose, threonine, leucine, thymine, thiamine and nalidixic acid were used

as selective plates. Plasmids pF'Lac and VR538 drdl (Su, Sm, Cm) were used for mobilization experiments.

Curing experiments

Antibiotic resistant, lactose fermenting and hemolytic strains were treated with acridine orange (AO) (Miller, 1972).

DNA-DNA hybridization

Plasmid DNAs were transferred from agarose gels to nitrocellulose (NC) membranes (Southern, 1975).

Bacterial colonies for hybridization studies were grown on NC membranes as described by Maniatis *et al.* (1982). Chromosomal DNA obtained by the method described by Mekalanos (1983) was also denatured and fixed on NC membranes.

Nick translations were performed with [α32P] - dATP (from the Instituto de Química-USP) as described by Maniatis *et al.* (1982).

The probes used were as follows: EcoRI/PstI fragment (754 bp), containing part of the ampicillin resistance gene (class C), from pBR322; *Ava* I/*Bam* HI fragment (1050 bp), containing part of the tetracycline resistance gene (class A) from pBR322 and a *Pvu*II fragment (322 bp), from pUC 19, containing part of the β-galactosidase gene of M13 phage.

Overnight hybridizations were performed in 2X SSC, 2 mM sodium pyrophosphate with 10^7 cpm of denatured DNA, under two different conditions of stringency, one at 45°C in 50% formamide and the other at 37°C in 30% formamide. The membranes were washed three times, for 30 min, in 2X SSC, 0.1% SDS, 2 mM sodium pyrophosphate at the same temperatures used for each hybridization and autoradiographed after 3-6 days.

RESULTS

Identification of plasmid-harboring-bacteria

Among a total of 420 colonies (15-25 from each animal) screened for plasmid content, 123 (29%) showed at least one distinct plasmid band on DNA agarose gel electrophoresis. Most isolates contained only a single band. Neverthess, some showed as many as five bands, ranging from approximately 3 to 130 MDa in size.

The 123 plasmid-carrying isolates were identified by biochemical tests. The distribution of bacterial genera in the nine amphibian species is shown in Table I. The

most prevalent bacterium was the species (*Citrobacter freundii*-Cf (67%), followed by *Enterobacter cloacae*-El (10%), *Klebsiella pneumoniae*-Kp (5%), *Escherichia coli*-Ec (3%), *Providencia alcalifaciens*-Pa (3%), *Enterobacter hafniae*-Eh (2%), *Enterobacter* sp-Es (2%), *Proteus rettgeri*-Pr (2%), *Aeromonas hydrophila*-Ah (2%), *Enterobacter aerogenes*-Ea (1%), *Enterobacter aglomerans*-Eg (1%), *Klebsiella oxytoca*-Ko (1%), *Salmonella* sp-Ss (1%). Two percent of the samples were not identified.

Susceptibility to antimicrobial agents

Data on antibiotic sensitivity of the bacterial isolates are shown in Table II. None was resistant to Nal, Cm and Km. Resistance pattern frequencies are given for each genus (Table III). For analyzing these patterns, only organisms which grew in the presence of the antibiotics at levels ≥ 10 µg/ml were considered resistant.

Table II - Incidence of Ap, Sm and Tc resistance in plasmid-harboring bacterial isolates.

Antibiotic concentration (µg/ml)	Ap		Sm		Tc	
	No.	%	No.	%	No.	%
1	22	17.9	50	40.7	114	92.7
5	50	40.7	65	52.8	1	0.8
10	18	14.6	8	6.5	2	1.6
20	15	12.2	-	-	-	-
50	9	7.3	-	-	4	3.3
100	7	5.7	-	-	2	1.6
500	2	1.6	-	-	-	-
Total	123	100.0	123	100.0	123	100.0

Hemolysin and fimbriae production

Two hemolytic ampicillin resistant strains, Ah 7.01 and Es 7.16, lost their plasmids after treatment with AO, though their hemolytic and ampicillin resistance properties remained intact.

Table III - Patterns of bacterial resistance to antibiotics, at concentrations of 10 μg or more per ml, distributed by genera.

Resistance patterns	*Citrobacter*	*Enterobacter*	*Klebsiella*	*Providencia*	*Proteus*	*Escherichia*	*Aeromonas*	*Salmonella*	Unidentified	Isolates	
										No.	%
A	53	1	3	-	-	2	-	1	2	62	50.4
B	22	15	4	-	-	2	2	-	-	45	36.6
C	6	-	-	-	-	-	-	-	-	6	4.9
D	-	-	-	4	-	-	-	-	-	4	3.2
E	2	-	-	-	-	-	-	-	-	2	1.6
F	-	2	-	-	2	-	-	-	-	4	3.2
Total	83	18	7	4	2	4	2	1	2	123	99.9

A - sensitive to all antibiotics tested; B - resistant to Ap; C - resistant to Sm; D - resistant to Tc; E - resistant to Ap and Sm; F - resistant to Ap and Tc.

Relationship between antibiotic resistance, lactose fermentation and plasmid content

Bacterial isolates harboring plasmids with a molecular weight higher than 23 MDa were grouped according to the similarities observed in their biochemical characteristics, antibiotic-resistance patterns and plasmid mobility profiles on agarose gels. Thirty antibiotic resistant and 34 lactose fermenting isolates were chosen and mated to *Escherichia coli* K12 C600. Yet, even when plasmids pF'Lac or VR538 drdl were used to mobilize them, no transconjugants demonstrating Ap, Tc, or Sm resistance or lactose fermentation were found. Colonies having lost their antibiotic resistance or lactose fermenting properties after AO treatment were not observed.

The presence of genes responsible for Tc or Ap resistance was also screened by DNA-DNA hybridization. Plasmid DNA from the 30 antibiotic resistant strains was transferred from the agarose gels to NC membranes and hybridized to Tc and Ap probes. No hybridization was detected in any of the assay conditions used.

Lac Z genes were searched for in the plasmids and in the chromosomes of the 34 lactose fermenting strains, using the 322 pb (PvuII fragment excised from pUC19) labeled probe. Among almost all the 34 isolates studied, hybridization was observed only with total DNA (Figure 1), but not with isolated plasmid DNA, even at low stringent assay conditions.

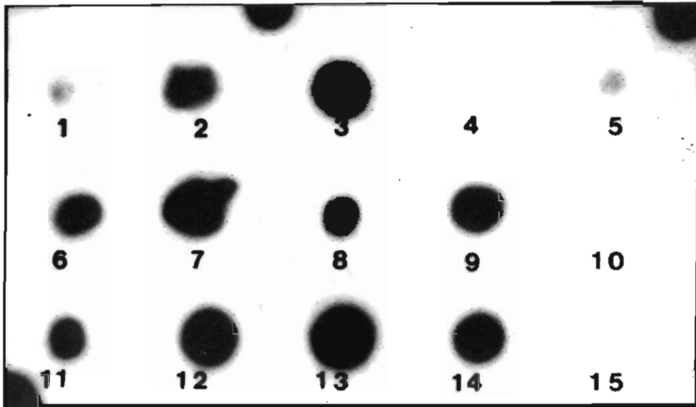

Figure 1 - Dot blot hybridization of the 322 bp, PvuII Lac fragment of pUC19, to total DNA of: the lactose fermenting strains (1-11) and strains used as positive (12-14) and negative (15) controls. 1) Eh 3.17; 2) Kp 3.01; 3) Kp 3.03; 4) Kp 9.03; 5) Ko 8.04; 6) Kp 3.07; 7) Ea 4.05; 8) Ec 3.03; 9) Cf 3.01; 10) Cf 4.22; 11) Ec 9.02; 12) *E. coli* TB1 (pVC2), Lac gene of *Streptococcus lactis*; 13) *E. coli* TB1 (pUC19); *E. coli* J53 Lac +; 14) *E. coli* J53 Lac +; 15) Pr 7.01 Lac - .

DISCUSSION

Among 420 colonies screened for plasmid content, (29%) showed at least one distinct plasmid band on agarose gels. This frequency fits patterns reported for plasmids observed in natural bacterial populations from different sources: soil, sea water, sea sediment, rivers, etc. (Christiansen *et al.*, 1973; Hada and Sizemore, 1981; Burton *et al.*, 1982; Glassman and MacNicol, 1984; Kobori *et al.*, 1984). The actual frequency could be higher than 29%, since some plasmid bands could be obscured by chromosomal DNA. The majority of isolates had a single plasmid yet some contained as many as five plasmid bands. Size classes were estimated by comparing the unknown plasmids' relative migration with the standard plasmids, used as molecular weight markers. The high frequency of plasmids with molecular weight higher than 23 MDa, was not in accordance with the reported surveys in naturally occurring strains, among which small plasmids were predominant (Jamieson and Bremmer, 1979; Hada and Sizemore, 1981; Glassman and MacNicol, 1984; Kobori *et al.*, 1984).

Although a complete study of bacterial flora was not performed, the results are in agreement with previous findings that *Citrobacter* is the predominant Enterobacteriaceae genus isolated from feces of cold-blooded animals (Moreno *et al.*, 1973; Roggendorf and Muller, 1976; Graves *et al.*, 1988). The biotypes found resembled those

encountered in *Rana* (van der Waaij *et al.*, 1974; Carr *et al.*, 1976) and other cold-blooded animals (Moreno *et al.*, 1973; Roggendorf and Muller, 1976).

Because of the observation of van der Waaij *et al.* (1974) that the aerobic bacterial flora present in the cloacae of *R. pipiens* is very similar to that identified in the insects used to feed them, the wild amphibians were not fed after being caught. They were sacrificed, as soon as they reached the laboratory and their feces immediately streaked onto MacConkey agar plates.

Some characteristics most frequently associated with the presence of plasmids were searched for in these isolates. The phenotypes were chosen based on the frequency data of association previously found in plasmids harboring enterobacteria strains (Moller *et al.*, 1978; Foster, 1983; Edwards and Ewing, 1986). Phenotypes which could be easily analyzed by conjugal transfer, transformation and curing, such as, drug resistance and sugar fermentation were preferentially investigated by these methods.

Antibiotic resistance is widespread among enterobacteria that cause disease in man and animals and usually is determined by plasmids (Anderson, 1968; Milch and Nguyen, 1981; Foster, 1983). The intensive use of antibiotics can explain the selection for R plasmids among bacteria of man and domestic animals, but not the incidence of R plasmids in naturally occurring bacteria. R plasmids were found in gram negative bacteria isolated from an antibiotic virgin community (Gardner *et al.*, 1969; Davis and Nandan, 1970) and from strains collected and stored in laboratories before the use of antibiotics (Hughes and Datta, 1983).

The presence of a considerable number of antibiotic resistant strains suggested the possible presence of plasmid-borne resistance. However, after a great number of mating and curing experiments to Ap and Tc resistance markers, the question remains unanswered.

The β-lactamase and Tc resistance genes were also screened for by DNA-DNA hybridizations, using class A probe for Ap resistance and class C probe for Tc resistance. It has been demonstrated that there are different determinants of Ap and Tc resistance. For Ap more than 20 biochemically different types have been described (Ambler and Scott, 1978; Mattew *et al.*, 1979; Boissinot *et al.*, 1987; Chang and Bolton, 1987; Kron *et al.*, 1987) which have been classified into three groups, A, B and C. However, the relationship between these β-lactamases has not been well established either immunologically or by hybridization. The type A (TEM-1) probe was chosen based on its distribution frequency among plasmids of gram negative bacteria. It was found in *Enterobacter*, *Proteus, Citrobacter* (Mattew, 1979), *Klebsiella* (Petit *et al.*, 1988) and *Aeromonas* (Chang and Bolton, 1987), species present among our isolates.

Tc resistance genes of R plasmids have been classified into five classes (A, B, C, D, and E) on the basis of DNA homology (Mendez *et al.*, 1980; Marshall *et al.*, 1986). The class C probe from pBR322 was used to screen for Tc genes since it has been found

in different genera of gram negative aerobic bacteria (Levy, 1984). Moreover, under low stringency, cross-hybridization between A, B, and C classes has been demonstrated (Marshall *et al.*, 1983). None of the isolates tested was found to hybridize with that probe. The probe showed homology to the plasmids RP4 (class A) and R124 (class B) used as positive controls.

Despite the fact that 110 (45%) of 244 lactose fermenting bacterial isolates harbored plasmids, while only 13 (7%) of 176 non-fermenting isolates were plasmid carriers, the expected Lac plasmids were not observed. The findings, in our experiments done with a considerable number of strains analyzed by mating, mobilization, curing and by DNA-DNA hybridizations, using a β-galactosidase probe, indicate that lactose fermentation is not mediated by plasmid genes, but by Lac genes present in the chromosomes of these bacteria.

ACKNOWLEDGMENTS

We thank Drs. Maria Nazareth R. Gay and Nelson Leon for their criticisms and suggestions concerning the manuscript, Drs. Maria Regina F. Toledo and Elza Mamizuka for providing confirmation and identification of the bacterial strains, Dr. Werner Bokerman for identifying the amphibians and Wolfgang Fisher for technical assistance.

This research was supported by Conselho nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Fundação Banco do Brasil (FBB) and Fundo Especial de Despesas do Instituto Butantan (FEDIB).

Publication supported by FAPESP.

RESUMO

Bactérias aeróbicas gram-negativas foram isoladas de fezes de 20 anfíbios. Os animais foram capturados na região que seria inundada para a formação da represa da Usina Hidrelétrica de Tucuruí-PA. Entre as 420 colônias bacterianas analisadas, 123 (29%) eram portadoras de plasmídios, sendo: 89% capazes de fermentar a lactose, 41% resistentes à ampicilina, 6,5% à estreptomicina e 6,5% à tetraciclina, em níveis ≥ 10 µg/ml. O gênero predominante entre as 123 amostras foi o *Citrobacter* (67%), seguido de *Enterobacter* (16%), *Klebsiella* (6%), *Providencia* (3%), *Proteus* (2%), *Aeromonas* (2%) e *Salmonella* (1%).

Como grande parte dos plasmídios detectados tinha pesos moleculares suficientes para conter genes capazes de promover sua auto-transferência, a correlação entre a presença dos mesmos e algumas características observadas foi investigada por conjugação. A presença destes genes foi também analisada por segregação natural e induzida, e por hibridização DNA-DNA, através de sondas radioativas.

Os resultados não demonstraram a presença de plasmídios R, Hly, ou Lac nestas amostras, mas foram encontradas evidências de que os caracteres de fermentação de lactose e produção de hemolisinas são mediados por genes presentes nos cromossomos destas bactérias.

REFERENCES

Affonso, M.H.T., Toledo, M.R.F. and Trabulsi, L.R. (1983). Natureza genética da fermentação de lactose em amostras de *Salmonella typhimurium*. *Rev. Microbiol.* *8*: 110-116.

Ambler, R.P. and Scott, G.K. (1978). Partial amino acid sequence of penicillinase coded by *Escherichia coli* plasmid R6K. *Proc. Natl. Acad. Sci.* (USA) *75*: 3732-3736.

Anderson, E.S. (1968). The ecology of transferable drug resistance in the enterobacteria. *Ann. Rev. Microbiol.* *22*: 131-180.

Boissinot, M., Mercier, J. and Levesque, R.G. (1987). Development of natural and synthetic DNA probes for OXA-2 and TEM-1 β-lactamases. *Antimicrob. Agents Chemother.* *31*: 728-734.

Burton, N.F., Day, M.J. and Bull, A.T. (1982). Distribution of bacterial plasmids in clean and polluted sites in South Wales River. *Appl. Environ. Microbiol.* *44*: 1026-1029.

Carr, A., Amborski, R.L., Culley Jr., D.D. and Amborski, G.F. (1976). Aerobic bacteria in the intestinal tracts of Bullfrogs (*Rana catesbiana*) maintained at low temperature. *Herpetologica* *32*: 239-244.

Chang, B.J. and Bolton, S.M. (1987). Plasmids and resistance to antimicrobial agents in *Aeromonas sobria* and *Aeromonas hydrophila* clinical isolates. *Antimicrob. Agents Chemother.* *31*: 1281-1282.

Christiansen, C., Christiansen, G., Bak, A.L. and Stenderup, A. (1973). Extrachromosomal deoxyribonucleic acid in different entero-bacteria. *J. Bacteriol.* *144*: 367-477.

Davis, C.E. and Nandan, J. (1970). The evolution of R factor. A study of preantibiotic community in Borneo. *New England J. Med.* *283*: 117-122.

Eckhardt, T. (1978). A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. *Plasmid* *1*: 584-588.

Edwards, P.R. and Ewing, W.H. (1986). *Identification of Enterobacteriaceae*. Elsevier Science Publishing Co. Inc. (4.ed.). New York, USA.

Foster, T.J. (1983). Plasmid determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol. Rev.* *47*: 361-409.

Gardner, P., Smith, D.H. and Beer, H. (1969). Recovery of resistance (R) factors from a drug-free community. *Lancet* 2: 774-776.

Glassman, D.L. and MacNicol, L.A. (1984). Plasmid frequency in natural populations of estuarine microorganism. *Appl. Environ. Microbiol.* *48*: 518.

Gossling, J., Loesche, W.L. and Nace, G.W. (1982). Large intestine bacterial flora of nonhibernating and hibernating Leopard frogs (*Rana pipiens*). *Appl. Environ. Microbiol.* *44*: 59-66.

Graves, S.R., Rawlinson, P.A., Kennelly-Merrit, S.A., MacLaren, D.A., Harvey, K.J. and Thorton, W.B. (1988). Enteric bacteria of reptiles on Java and the Krakatau Islands. *Phil. Trans. R. Soc. Lond. B* *322*: 355-361.

Gross, D.C., Vidaver, A.K. and Keralis, M.B. (1979). Indigenous plasmids from phytopathogenic *Corynebacterium* species. *J. Gen. Microbiol.* *115*: 479-489.

Hada, H.S. and Sizemore, R.K. (1981). Incidence of plasmids in marine *Vibrio* spp. isolated from an oil field in the northwestern gulf of Mexico. *Appl. Environ. Microbiol.* *41*: 199-202.

Hughes, V. and Datta, N. (1983). Conjugative plasmids in bacteria of the pre-antibiotic era. *Nature* *302*: 725-727.

Iveson, J.B., Mackay-Scollay, E.M. and Bamford, V.W. (1969). *Salmonella* and *Arizona* in reptiles and in man in Western Australia. *J. Hyg. Camb. 67*: 135-145.

Jamieson, A.F. and Bremmer, D.A. (1979). Characterization of plasmids from antibiotic resistant *Shigella* isolates by agarose gel electrophoresis. *J. Gen. Microbiol. 117*: 73-81.

Kado, C.I. and Liu, S.T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol. 145*: 1365-1373.

Kobori, H., Sullivan, C.W. and Shizuya, H. (1984). Bacterial plasmids in Antartic natural microbial assemblages. *Appl. Environ. Microbiol. 48*: 515-518.

Kron, M.A., Currie-McCumber, D.M.C. and Medeiros, A.A. (1987). Molecular epidemiology of OHIO-1 β-lactamase. *Antimicrob. Agents Chemother 31*: 2007-2009.

Levy, S.B. (1984). Resistance to the tetracyclines. In: *Antimicrobial drug resistance* (Bryan, L., ed.). Academic Press, Orlando, Fl.

Lins, Z.C. (1971). Studies on enteric bacterias in the lower Amazon region: II *Salmonella* types isolated from wild reptiles in Pará state-Brazil. *Rev. Microbiol.* 2: 165-169.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). *Molecular cloning. A laboratory manual.* Cold Springer Harbor Laboratory, Cold Spring Harbor, New York - USA.

Marshall, B., Tachibana, C. and Levy, S.B. (1983). Frequency of tetracycline resistance determinant classes among lactose-fermenting coliforms. *Antimicrob. Agents Chemoter 24*: 835-840.

Marshall, B., Morrissey, S., Tlynn, P. and Levy, S.B. (1986). A new tetracycline-resistance determinant, class E, isolated from Enterobacteriaceae. *Gene 50*: 111-117.

Mattew, M., Hedges, R.W. and Smith, J.T. (1979). Types of β-Lactamase determined by plasmids in gram negative bacteria. *J. Bacteriol. 138*: 657-662.

Mattew, M. (1979). Plasmid-mediated β-lactamase of gram negative bacteria: properties and distribution. *J. Antimicrob. Chemother. 5*: 349-358.

Mekalanos, J.J. (1983). Duplication and amplification oftoxin genes in *Vibrio cholerae. Cell 35*: 253-263.

Mendez, B., Tachibana, C. and Levy, S.B. (1980). Heterogeneity of tetracycline resistance determinants. *Plasmid 3*: 99-108.

Milch, H. and Nguyen, T.K. (1981). *Klebsiella* and *Enterobacter* strains derived from hospital infections. *Acta Microbiol. Acad. Sci. Hung. 28*: 171-196.

Miller, J.H. (1972). Curing of episomes from *Escherichia coli* strains with acridine orange. p. 104-106. In: *Experiments in molecular genetics* (Cold Spring Harbor Lab., ed). Cold Spring Harbor, New York.

Moller, R.K., Jorgensen, N.H.F., Christiansen, C., Christiansen, G., Bak, A.L. and Stenderup. A. (1978). Characterization of plasmids from wild type Enterobacteriaceae, p. 257-261. In: *Microbiology* (D. Schiessinger, ed.). American Society for Microbiology, Washington, DC.

Moreno, G., Lopez, C.A.M., Belluomini, H.E., Pessoa, G.V.A., Biasi, P. and Andrade, J.C.R. (1973). Enterobactérias isoladas de anfíbios e répteis. *Rev. Inst. Med. Trop. 15*: 122-126.

Petit, A., Sirot, D.L., Chanal, C.M., Sirot, J.L., Labia, R., Gerbaud, G. and Cluzel, R.A. (1988). Novel plasmid-mediated β-Lactamases in clinical isolates of *Klebsiella pneumoniae* more resistant to ceftazidine than to other broad-spectrum cephalosporins. *Antimicrob. Agents Chemother 32*: 626-630.

Roggendorf, M. and Muller, H.E. (1976). Enterobacteria of reptiles. *Zbl. Bakt. I. Abt. orig. A. 236*: 22-35.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol. 98*: 503-517.

van der Waaij, D., Cohen, B.J. and Nace, G.W. (1974). Colonization pattern of aerobic gram-negative bacteria in the cloacae of *Rana pipiens. Lab. Anim. Sci. 24*: 307-317.

von Wright, A., Suominen, M. and Sivela, S. (1986). Identification of lactose fermentation plasmids of streptococcal dairy starter strains by Southern hybridization. *Letters in Appl. Microbiol. 2*: 73-76.

Willets, N. and Wilkins, B. (1984). Processing of plasmid DNA during bacterial conjugation. *Microbiol. Rev. 48*: 24-41.

Zahner, H. and Maas, W.K. (1972). *Biology of antibiotics*. (Springer Verlag Inc., ed.). New York.

(Received November 19, 1990)