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Identification of multiple-resistance (R) and colicinogeny (Col) plasmids in an epidemic *Salmonella agona* serotype in Rio de Janeiro

BY ANA C. P. VICENTE AND D. F. DE ALMEIDA

Laboratório de Fisiologia Celular, Instituto de Biofísica, Universidade Federal do Rio De Janeiro, 21.941, *Rio de Janeiro, RJ, Brasil*

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SUMMARY

A *Salmonella agona* strain has caused a hospital outbreak of gastroenteritis in a pediatric unit in Rio de Janeiro. It bears two plasmids, a small (6·5 MDa molecular weight) plasmid coding for type B colicin production and a larger one (36 MDa molecular weight) determining resistance to ampicillin, gentamicin, kanamycin and trimethoprim-sulphamethoxazole. The R-plasmid, but not the Col-plasmid, is self-transferable to a *Escherichia coli* recipient strain. Curing for the R-plasmid was achieved by treatment with 0·05 % SDS followed by incubation at 44 °C. It has not been possible to cure the *S. agona* strain for its Col-plasmid.

INTRODUCTION

Since its first appearance as a major epidemic serotype, *Salmonella agona* has occupied a prominent position worldwide among salmonella isolates from man and animals (Clark *et al.* 1973; Barker, Old & Tyc, 1982). In Brazil (Rio de Janeiro) the occurrence of *S. agona* was first reported among salmonella isolates from sewage obtained between 1976 and 1978 (Câmara *et al.* 1982). More recently, through a national survey, Solari (1983) has collected 240 isolates of *S. agona* from human, foods and environmental sources. These include a *S. agona* serotype which caused a hospital outbreak of gastroenteritis at the Childrens Hospital, Federal University of Rio de Janeiro, in 1979.

This paper reports the presence of multiple drug resistance and colicinogeny in the epidemic *S. agona* strain, and describes some genetic properties of the corresponding R- and Col-plasmids.

MATERIALS AND METHODS

Bacterial strains

The 13 isolates of salmonella were from a diarrhoeal disease outbreak at the Childrens Hospital, Federal University of Rio de Janeiro. All strains were recovered from faeces. Isolation and determination of the resistance pattern were performed by I. Ricciardi, Institute of Microbiology, Federal University of Rio de

Janeiro. Serotypes were determined at the Central Public Health Laboratory, Colindale, London.

Media and growth conditions

Cultures were grown aerobically at 37 °C in YET medium made up of (in g/l): tryptone, 10; yeast extract, 5; sodium chloride, 5; pH 7·2. McConkey medium (Merck) was used for transconjugant selection. When necessary, 100 μg/ml nalidixic acid (Sigma) and/or 20 μg/ml antibiotics (ampicillin, gentamicin or kanamycin; Bristol Laboratories) were added.

Colicin

Cultures were examined for colicin production by the overlay method (Ozeki, Stocker & Smith, 1962). The type of colicin was determined by the use of a set of colicin-resistant *Escherichia coli* indicator strains, listed in Table 1.

Table 1. *Strains used for colicin typing*

Indicator strains	Designation
22R80	*E. coli* K12 Row (standard colicin-sensitive strain)
22R966	*E. coli* K12 Row B^r
20R675	*E. coli* K12 Col E1
20R676	*E. coli* K12 Col E2
22R81	*E. coli* K12 Col I, V^r
22R82	*E. coli* K12 Col Ia
22R83	*E. coli* K12 Col Ib
20R914	*E. coli* K12 Row K^r
20R915	*E. coli* K12 B^r, V^r

These strains were kindly provided by B. Rowe, Central Public Health Laboratory, Colindale, London.

Plasmid transfer

The mating technique consisted in mixing exponential phase donor and recipient cultures (1:10). The recipient was the nalidixic acid-resistant *E. coli* strain LR1 (Câmara *et al.* 1982). After incubation at 37 °C for 3 or 24 h, samples (0·1 ml) of appropriate dilutions were spread on McConkey agar plates containing nalidixic acid and the selective drug (ampicillin, gentamicin or kanamycin), as required. A total of 300 transconjugants per experiment, picked at random from the three selective plates, were screened for colicin production.

Plasmid DNA isolation

The method of Kado & Liu (1981) was used. The resulting DNA solution was submitted to electrophoresis (12 V . cm^{-1}) on 0·7 % agarose gel. Buffer composition (g . l^{-1}) was: Tris, 1·52; EDTA, 0·9; boric acid, 0·5; pH 7·8.

Plasmid curing

Treatment of cultures with ethidium bromide (Bouanchaud, Scavizzi & Chabbert, 1969), acridine orange (Watanabe & Fukasawa, 1961) or SDS/high temperature (Hill & Carlisle, 1981) were the methods used to obtain plasmid curing.

Transformation

Ten ml of a recipient culture grown for 3 h at 37 °C was centrifuged (5400 **g**, 5 min), resuspended in half volume of 0·05 M-$CaCl_2$, kept for 15 min at 0 °C, centrifuged again under the same conditions, and resuspended in 1/10 volume of 0·05 M-$CaCl_2$ at 0 °C. Plasmid DNA (25 μl of a 2 $\mu g.ml^{-1}$ solution) was added to 0·2 ml of the cell suspension. The mixture was incubated successively for 15 min at 0 °C, 10 min at 32 °C and 15 min at 0 °C. YET medium (0·3 ml) was added and incubation carried out at 37 °C for 90 min. Suitable dilutions were spread on solid medium for the selection of col^+ transconjugants.

RESULTS

Resistance pattern and resistance transferability

The isolates, all of the *S. agona* serotype, were resistant to ampicillin, gentamicin, kanamycin and trimethoprim-sulphamethoxazole.

Conjugational transfer to a recipient *E. coli* strain was observed after 3 h at 37 °C. Frequencies of transfer ranged from 10^{-5} to 7×10^{-6}, depending on individual isolates. Irrespective of the drug used for selection (ampicillin, gentamicin or kanamycin) the three resistance markers were always cotransferred to the recipient strain.

Colicin production and identification

Out of the 13 drug-resistant *S. agona* strains, seven (54 %) were colicinogenic. In each case, type B colicin was produced. Attempts to transfer the col^+ character to a recipient *E. coli* strain were not successful after 3 h conjugation. Prolonging conjugation time to 24 h resulted in the obtention of 3–11 % colicinogenic organisms among the drug-resistant transconjugants (Table 2).

Table 2. *Incidence of colicinogeny* (col^+) *among drug-resistant* E. coli *transconjugants*

Donor *S. agona* strain	Frequency of transfer* of selected marker			Frequency of col^+ character‡ among transconjugants (%)
	A†	G	K	
31-3	$4 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	$6 \cdot 10^{-4}$	9
33-8	$5 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	10
69-2	$8 \cdot 10^{-4}$	$8 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	4
75-4	$1 \cdot 10^{-5}$	$2 \cdot 10^{-5}$	$1 \cdot 10^{-5}$	5
76-9	$5 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	$1 \cdot 10^{-4}$	3
95-4	$1 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	3
96-7	$1 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	11

* The frequency of transfer is defined as the ratio between the number of transconjugants found after 24 h at 37 °C and the initial number of donor cells.

† Abbreviations: A, ampicillin; G, gentamicin; K, kanamycin.

‡ A total of 300 transconjugants taken at random from the three selective plates were tested for colicinogeny in each experiment.

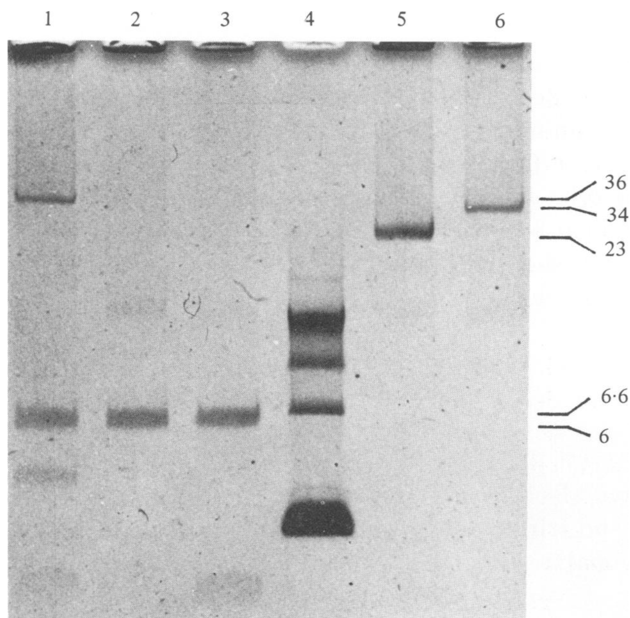

Fig. 1. Electrophoretic analysis of plasmid DNA of *Salmonella agona* and *Escherichia coli* strains using 0·7 % agarose. Lanes: 1, *Salmonella agona* (strain 95·4); 2, *Escherichia coli* LR1 transformed to *col*$^{+}$; 3, *S. agona col*$^{+}$ (strain 95·4), cured for the resistance markers; 4, 5, and 6, standard-molecular-weight plasmids. Sizes of DNA in Megadaltons are given on the right.

Plasmid DNA isolation

Electrophoretic analysis revealed the presence of two plasmids in the colicinogenic, drug-resistant *S. agona* strains, one of 36 MDa and the other of 6·5 MDa molecular weight (Fig. 1).

Plasmid curing

Curing experiments revealed that the 36 MDa molecular weight plasmid was associated with the multiple drug resistance (Fig. 1). Methods routinely used for bacterial plasmid elimination, such as treatment with acridine orange or ethidium bromide had no effect when applied to the drug-resistant *S. agona* strains. Curing the R-plasmid at a frequency of 2–10 % has been achieved however using the Hill & Carlisle (1981) technique of consecutive treatments with 0·05 % SDS and high temperature (44 °C). In no case has the *col*$^{+}$ character been abolished.

Transformation

A DNA preparation extracted from a colicinogenic *S. agona* strain was used to transform *E. coli* LR1. Transformants have been obtained which were colicin B producers. A plasmid DNA preparation from a transformant produced a single, 6·5 Mda molecular weight DNA band on agarose gel electrophoresis, indicating that the *col*$^{+}$ character of the *S. agona* strains is determined by the smaller of the two plasmids (Fig. 1).

DISCUSSION

An international collection of 419 isolates of *S. agona* from human, domestic animals and environmental sources revealed a total of 68 (16·2 %) colicinogenic strains (Barker, Old & Tyc, 1982). A similar frequency of colicinogeny (about 12 %) has also been reported among *S. typhimurium* strains (Barker, 1980). Screening the *S. agona* collection from the recent national survey conducted by Solari (1983) we found 16·6 % (40/240) colicinogenic strains. An interesting finding resulting from that survey refers to the isolates from humans. They numbered 13 (32·5 % of the col^{+} strains), originated from distinct geographical regions of the country; 12 (92 %) were colicinogenic, which include the seven strains referred to in this paper. It should also be noted that 91·6 % (11/12) of the col^{+} *S. agona* of human origin produced type B colicin. This contrasts with findings from the northern hemisphere, which reveal a rather low incidence of type B colicin production among salmonella isolates (Barker, 1980; Barker *et al.* 1982). We have not investigated the possible association of colicin M production with the presence of colicin B, as sometimes observed (Fredericq & Smarda, 1970; Sasarman *et al.* 1980).

Colicinogeny may be a significant factor in determining the course of gastro-intestinal infections (Hardy, 1975; Bures, Horak & Duben, 1979). Furthermore, colicinogeny may occur in association with drug-resistance, either on the same plasmid (R. Col) or on different molecules (Sasarman *et al.* 1980). In the present case, colicinogeny and drug-resistance are mediated by two distinct plasmids. Based on transformation and on plasmid curing experiments we conclude that the small (6·5 MDa) plasmid determines colicinogeny, whereas the 36 MDa plasmid codes for the multiple drug resistance. The R-plasmid is self-transferable, and is probably responsible for the mobilization of the Col plasmid. The small molecular weight of the latter precludes the existence of the genes needed for self-transfer; the smallest naturally occurring self-transferable plasmids have sizes of about 25 MDa (Broda, 1979).

A final point should be made about the application of a non-conventional method for plasmid curing, consisting of successive treatments with 0·5 % SDS and high temperature (44 °C) incubation (Hill & Carlisle, 1981). These two modes of curing bacterial plasmids were applied during attempts to enrich for *E. coli* strains present in foods as contaminants (Hill & Carlisle, 1981). Combined treatment seems to be a more efficient method of plasmid curing than either detergent or temperature alone, although it has not been successful in eliminating the Col B plasmid.

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