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Characterization of R-plasmids coding for ampicillin resistance from *Salmonella* species

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SUMMARY

A sudden increase in the incidence of ampicillin resistance was observed among *Salmonella* species isolated within New Zealand in 1973–4. This increase was due mainly to the appearance and proliferation of *Salmonella newington* and *Salmonella anatum* serotypes resistant to ampicillin. The plasmid complements of 14 ampicillin-resistant *S. newington* and *S. anatum* isolates obtained from widely separated geographical areas within New Zealand between 1973 and 1974 were characterized by agarose gel electrophoresis. Each contained one or more plasmids ranging in molecular weight from 1·4 to 42 Mdal. Eleven isolates contained a self-transmissible plasmid of 33 Mdal which encoded resistance to ampicillin. After transfer to *Escherichia coli*, the 33 Mdal R-plasmids from each of these isolates were shown to be identical by restriction endonuclease analysis. The remaining three strains contained ampicillin R-plasmids having molecular weights of 35, 37·5 and 42 Mdal. These plasmids were shown by restriction endonuclease analysis to be related to the 33 Mdal R-plasmid. We conclude that the 33 Mdal plasmid and its derivatives were responsible for the increase in the incidence of ampicillin-resistant *S. newington* and *S. anatum* serotypes among the total *Salmonella* population.

INTRODUCTION

The use of the antibiotics ampicillin and its derivative amoxycillin has increased steadily since their introduction to New Zealand in 1963 and 1974 respectively. In 1973–4 there was a sudden marked increase in the proportion of *Salmonella* isolates which were resistant to ampicillin. This report describes the isolation and molecular characterization of self-transmissible R-plasmids associated with ampicillin resistance in these strains.

METHODS

Salmonella isolates were obtained from the New Zealand National Health Institute, Wellington, the national reference centre for *Salmonella*. They constitute a representative sampling of ampicillin-resistant *Salmonella* species isolated in New Zealand in 1973 and 1974. All were shown to be resistant to $> 32\ \mu g$ of

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ampicillin per ml using the agar dilution method described by Ericsson & Sherris (1971). Resistance to other antibiotics was investigated using the disk diffusion method described by Ericsson & Sherris (1971). None of the isolates were resistant to tetracycline, streptomycin, sulphafurazole, kanamycin, chloramphenicol, cephaloridine, gentamicin, trimethoprim or naladixic acid.

Plasmids were transferred from *Salmonella* to *Escherichia coli* K12 by conjugation with a naladixic acid-resistant derivative of J5-3 (F^- *met-63, pro-22*), as described by Bremner (1978). PB501 (F^-, *galT12*) was used as the recipient in K12 × K12 crosses.

Plasmid DNA was prepared by the method of Meyers *et al.* (1976). Care was taken to prevent shearing of chromosomal DNA before its removal by differential salt precipitation. This precaution significantly reduced the concentration of chromosomal DNA present in cleared lysates. Linear and open circular (OC) DNA was distinguished from covalently closed circular (CCC) DNA by removal of OC and linear DNA by acid phenol extraction as described by Zasloff, Ginder & Felsenfeld (1978).

Plasmid DNA was digested by endonuclease R: *EcoR1* and endonuclease *Pst1* at 37 °C for 1 h in the following reaction mixtures. For *EcoR1*: 20 μl of DNA (in 10 mM Tris pH 8·0, 1 mM EDTA), 2 μl of concentrated enzyme buffer (0·9 M Tris pH 7·8, 0·1 M-$MgCl_2$), 2 μl of *EcoR1* (1 unit/μl, prepared as described by Tanaka & Weisblum, 1975). For *Pst1*: 20 μl of DNA (in 10 mM Tris pH 8·0, 1 mM EDTA), 2 μl of concentrated enzyme buffer (0·5 M-NaCl, 0·06 M-$MgCl_2$, 0·06 M Tris pH 7·4, 0·06 M 2-mercaptoethanol, 1 mg/ml gelatin), 1 μl of *Pst1* (20 units/μl, a gift from Dr J. Bedbrook, Cambridge, England, to P. L. Bergquist).

Agarose gel electrophoresis was carried out in a horizontal gel apparatus similar to that described by McDonell, Simon & Studier (1977) for 3 h at 135 V in 0·7 % (w/v) or 0·85 % (w/v) agarose (Sigma A6877) in borate buffer (Meyers *et al.* 1976).

After electrophoresis, gels were placed on a Blak-Ray u.v. transilluminator screen (Ultraviolet Products Inc., San Gabriel, California 91778) and photographed with Agfapan 400 film which was developed using a reversal process.

RESULTS

Incidence of ampicillin resistance among Salmonella *serotypes in New Zealand*

Routine surveillance of *Salmonella* species for antibiotic resistance revealed an increase in the incidence of resistance to ampicillin in 1974 (Table 1, column 2). This increase was due primarily to the appearance and proliferation of ampicillin-resistant derivatives of the closely related *Salmonella newington* and *anatum* serotypes (Table 1, column 4). These two serotypes differ only by the presence of phage Epsilon 15 in *S. newington* and are often found in close association. The increase in the proportion of *S. newington* and *S. anatum* isolates resistant to ampicillin (Table 1, column 5) was paralleled by an increase in the incidence of these serotypes among the total *Salmonella* population (Table 1, column 6).

Table 1. *Incidence of ampicillin resistance among* Salmonella *isolates*

Year of isolation	No. of *Salmonella* tested for resistance to ampicillin	Proportion of *Salmonella* resistant to ampicillin	No. of *S. newington* and *S. anatum* serotypes screened	Proportion of ampicillin-resistant *S. newington* and *S. anatum* among the total *Salmonella* population (%)	Proportion of *S. newington* and *S. anatum* resistant to ampicillin (%)	Proportion of *S. newington* and *S. anatum* among the total *Salmonella* population (%)
1972	259	4·7	19	0·4	5·3	7·3
1973	274	5·2	30	1·7	16·7	11·0
1974	395	9·4	49	7·6	61·5	12·4
1975	387	3·9	12	1·0	33·3	3·1
1976	327	1·8	7	0·6	27·5	2·1
1977	419	3·6	23	0·5	8·7	5·5

Molecular characterization of plasmids in ampicillin-resistant S. newington *and* S. anatum *isolates*

Fourteen isolates of *S. newington* and *S. anatum* resistant to ampicillin were tested for the presence of plasmid DNA. Cleared lysates from each strain were electrophoresed in agarose gels. Each strain was shown to contain one or more plasmids having molecular weights ranging from 1·4 to 42 Mdal (Table 2). Molecular weights of plasmids were calculated by comparison of their electrophoretic mobilities with the mobilities of plasmids of known molecular weight shown in Plate 1.

All 14 isolates transferred ampicillin resistance to *Escherichia coli* K12 (J5-3).

Table 2. *Properties of ampicillin-resistant* Salmonella *isolates*

Strain	Year of isolation	Place of isolation*	Serotype	Molecular weight of ampicillin plasmid (Mdal)	Molecular weight of other plasmids present (Mdal)
3082	1973	Wn	*newington*	33	5·5, 2·3, 2·0
3317	1973	Ak	*newington*	33	2·3
3370	1973	Ak	*newington*	33	2·3
0097	1974	Wn	*newington*	33	5·5, 2·3, 2·0
0602	1974	Wn	*newington*	33	—
0681	1974	PN	*newington*	33	2·3, 2·0, 1·4
1133	1974	Ak	*newington*	33	2·0
1847	1974	Wn	*anatum*	33	3·3
1858	1974	Wn	*newington*	33	5·5, 2·3, 2·0
0745	1973	Ak	*anatum*	33	3·3
0692	1974	Chch	*newington*	33	2·3, 2·0, 1·4
1101	1974	Wn	*newington*	35	—
0278	1974	Ak	*newington*	37·5	—
3341	1973	Wn	*anatum*	42	17, 15, 3·3, 2·3, 2·15, 2·0

* Wn, Wellington; Ak, Auckland; PN, Palmerston North; Chch, Christchurch.

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Electrophoresis of cleared lysates from the exconjugants demonstrated that a single plasmid could be transferred in each case. The molecular weight of the ampicillin R-plasmid in the exconjugant always corresponded to the molecular weight of one of the plasmids in the donor cells. This result contrasts with those obtained on transfer of gentamicin and trimethoprim R-plasmids from clinical isolates to *E. coli* as often only part of these latter plasmid molecules are transferred (Jamieson & Bergquist, unpublished). The plasmids present in one of the *Salmonella* isolates and its exconjugants are shown in Plate 1. Ampicillin-resistant exconjugants resulting from crosses of 11 of the *Salmonella* isolates with *E. coli* all contained a plasmid with a molecular weight of 33 Mdal. These plasmids were self-transmissible and could be transferred to a second strain of *E. coli* (PB501). In a similar fashion, the remaining *Salmonella* isolates were demonstrated to contain self-transmissible plasmids encoding ampicillin resistance. These plasmids had molecular weights of 35, 37·5 and 42 Mdal. Additional estimates of the molecular weights of the R-plasmids were obtained by comparison of the mobilities of their linear fragments produced on digestion by the restriction endonucleases *EcoR1* and *Pst1* with the markers derived from *EcoR1* digestion of R100-1 (see Plates 2 and 3).

Most of the other plasmids present in the *Salmonella* isolates had molecular weights of less than 15 Mdal. As 15 Mdal of DNA is required to code for all functional conjugation systems studied so far (Achtman & Helmuth, 1975) it is unlikely that plasmids with molecular weights considerably less than 15 Mdal are self-transmissible. A proportion of the exconjugant cells from several crosses were detected which contained plasmids smaller than 15 Mdal together with a self-transmissible R-plasmid. We conclude therefore that transfer of the small plasmids was mediated by the larger R-plasmids.

Restriction endonuclease analysis of ampicillin R-plasmids

Comparison of the numbers and sizes of the DNA fragments produced by digestion of any two plasmids by a sequence-specific nuclease can be used as a measure of the relatedness of the plasmids (Thompson, Hughes & Broda, 1974). The degree to which the independently isolated ampicillin R-plasmids are related was determined by digestion of R-plasmid DNA from cleared lysates of ampicillin-resistant exconjugants with the restriction endonucleases *EcoR1* and *Pst1*. The 11 33 Mdal plasmids all gave identical sets of fragments on digestion with either *EcoR1* or *Pst1*. Examples of these fragment patterns are given in Plate 2, Wells B, B′ and C′ (*EcoR1* digests) and in Plate 3, well B (*Pst1* digest). We conclude that the 33 Mdal plasmids present in these 11 independent isolates are identical. The fragment patterns generated on digestion of the remaining three plasmids with *EcoR1* were different from each other and from those generated on *EcoR1* digestion of the 33 Mdal plasmid. However, each of these plasmids had several *EcoR1* fragments in common with the 33 Mdal plasmid (Plate 2). A similar result was obtained on digestion of two of these plasmids with *Pst1* (Plate 3).

Recent evidence has shown that segments of plasmid DNA having little or no ancestral relationship can be joined together by site-specific non-homologous

recombination involving insertion of IS sequencies (Cohen, 1976). It seems likely that the three larger R-plasmids described above evolved by insertion of DNA into the more common 33 Mdal plasmid.

For example, the R-plasmid in isolate 0278 was cleaved at six sites by *Pst1*. Five of the fragments produced had molecular weights corresponding to the five fragments generated on digestion of the 33 Mdal plasmid with *Pst1* (Plate 3). Hence the remaining fragment would appear to be integrated into a 33 Mdal-type plasmid at or near a *Pst1* site.

DISCUSSION

Aserkoff & Bennett (1969) have demonstrated that administration of ampicillin in salmonellosis favours the acquisition of plasmid mediated ampicillin resistance by the infecting strain. The use of ampicillin has increased rapidly in New Zealand since 1968 and it is virtually certain that for several years cases of salmonellosis were treated with this drug. Our results indicate that the increase in the incidence of ampicillin resistance in *Salmonella* isolates observed in New Zealand in 1974 was due to the acquisition of a 33 Mdal ampicillin R-plasmid by *S. newington* and *S. anatum* strains, followed by the subsequent proliferation of these strains possessing the selective advantage of resistance to ampicillin. Ampicillin is no longer recommended in the treatment of salmonellosis since its administration has been shown to have little effect on the course of the infection and indeed, prolongs the duration of faecal excretion of salmonellas (Aserkoff & Bennett, 1969). We suggest that an important factor in the reduction in the incidence of ampicillin resistance in *Salmonella* isolates observed in New Zealand since 1974 has been the cessation of the use of ampicillin for this purpose.

The 42, 37·5 and 35 Mdal ampicillin R-plasmids provide examples of *in vivo* plasmid evolution. Comparison of the sequence-specific nuclease digests of these plasmids to that of the more common 33 Mdal plasmid indicate that they most likely evolved by insertion of DNA into this latter plasmid. An obvious source of the inserted DNA is the small plasmids shown to be present in the majority of the *Salmonella* isolates that we have screened.

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EXPLANATION OF PLATES

PLATE 1

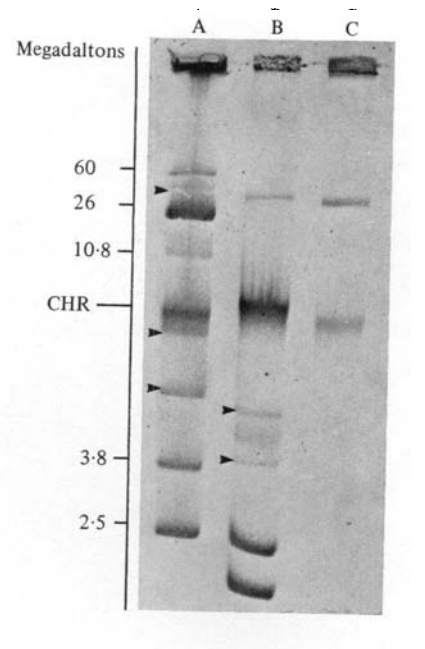
Electrophoresis in 0·7 % agarose of DNA from the ampicillin-resistant *Salmonella* isolate 3082. Open circular DNA is indicated by small arrows. CHR indicates the banding position of chromosomal DNA. (A) DNA standards. R100-1 (60 Mdal), R6K (26 Mdal), pML31 (10·8 Mdal) pMB9 (3·8 Mdal), pBR322 (2·5 Mdal). (B) DNA from a cleared lysate of isolate 3082. (C) DNA from a cleared lysate of an ampicillin-resistant exconjugant from the cross 3082 × *E. coli* J5-3.

PLATE 2

Electrophoresis in 0·85 % agarose of *EcoR1* digests of representative ampicillin R-plasmids from ampicillin-resistant *E. coli* exconjugants. The exconjugants were derived from crosses with ampicillin resistant *Salmonella* isolates. The DNA in well D′ includes the OC and CCC forms (arrowed) of a 2·0 Mdal plasmid present in the Ap^R exconjugant. This small plasmid was not cut with *EcoR1* and its CCC form obscures the 1·8 Mdal fragment produced on cleavage of the 42 Mdal plasmid. (A) R100-1 DNA digested with *EcoR1*. Molecular weights of R100-1 fragments are from Chandler *et al.* (1977). (B–D) *EcoR1*-digested DNA of the ampicillin plasmids of *Salmonella* strains 1858 (33 Mdal), 0278 (37·5 Mdal) and 1101 (35 Mdal) respectively. (A′) *EcoR1*-digested R100-1 DNA. (B′–D′) *EcoR1*-digested DNA of the ampicillin plasmids of *Salmonella* strains 3082 (22 Mdal), 3317 (33 Mdal) and 3341 (42 Mdal) respectively.

PLATE 3

Electrophoresis in 0·85 % agarose of *Pst1* digests of ampicillin R-plasmids from ampicillin resistant *E. coli* exconjugants derived from crosses with the *Salmonella* isolates. The small 0·4 Mdal fragment common to each is arrowed. (A) *EcoR1*-digested R100-1 DNA. Molecular weights of R100-1 fragments are from Chandler *et al.* (1977). (B–E) *Pst1* digests of ampicillin R-plasmids from *Salmonella* isolates. (B) 1858 (33 Mdal). (C) 0278 (37·5 Mdal). (D) 0692 (33 Mdal). (E) 1101 (35 Mdal).



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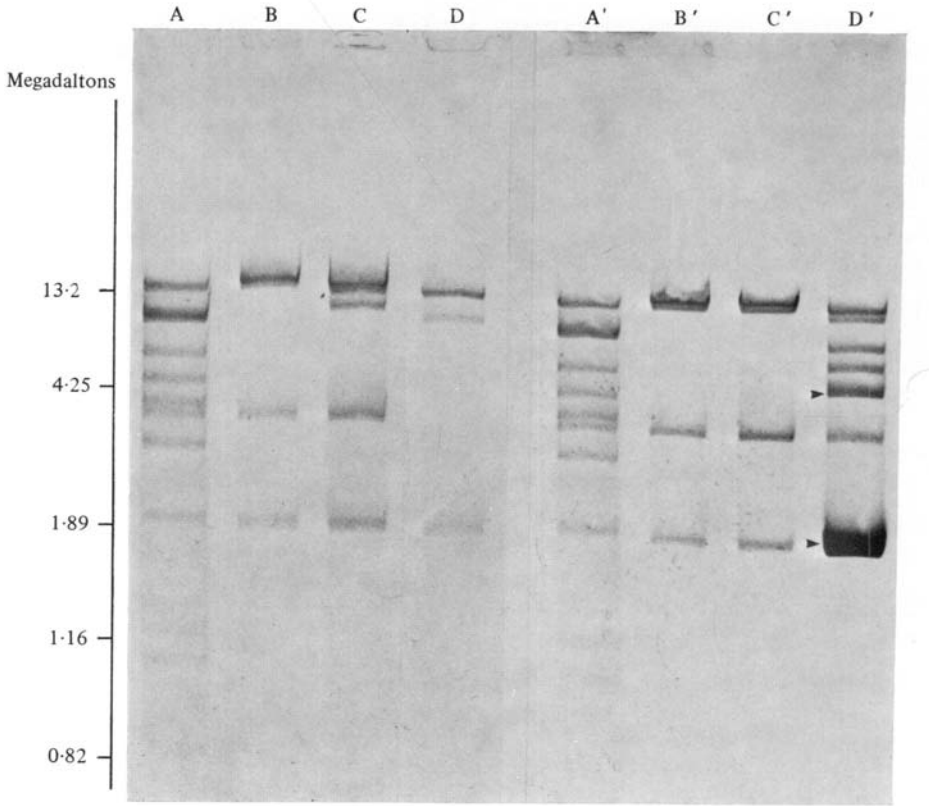

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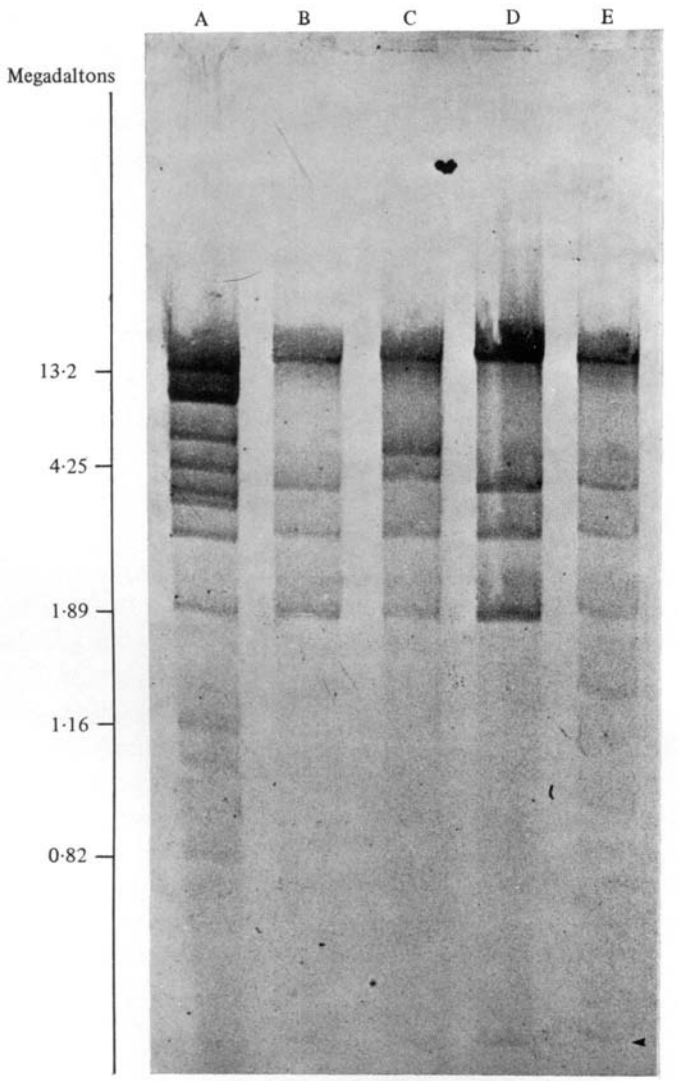
Plate 3



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