Genetic evidence for a chromosomally integrated multiresistance plasmid in *Salmonella dublin*

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Summary. Of 1099 isolates of *Salmonella dublin* during 1985–86, 11 (1%) were resistant to three or more antibiotics. Strain S4659/85, a multiresistant isolate, lacked autonomous R plasmids but showed incompatibility with *incH2* plasmids and donated resistance determinants in matings. Transconjugants acquired incomplete R plasmids which integrated stably into a specific chromosomal site. These data provide an insight into the behaviour of R plasmids in *S. dublin*.

Introduction

During the period 1985–86, *Salmonella dublin* was the second most common serotype isolated from cattle in Great Britain (Anon, 1985; Wray, 1985 and unpublished data). Although antibiotic multiresistance is uncommon in *S. dublin*, most bovine isolates of *S. typhimurium* and 70% of continental isolates of *S. dublin* are multiresistant (Helmuth and Seiler, 1986; Jorgensen, 1986; Sojka *et al.*, 1986). Since British isolates are subjected to the same antibiotic selective pressures as the multiresistant strains of *Salmonella*, it is possible that they are refractory to plasmids common to the enteric bacterial pool.

This paper reports a survey of antibiotic sensitivities of *S. dublin* isolates and presents evidence for chromosomal integration of a multiresistant conjugative *incH2* plasmid in one isolate.

Materials and methods

Strains and plasmids

Escherichia coli K12 marker strains were J62.1 F⁻ *lac pro his trp nalA*, C600 F⁻ *lac thr leu thi supE tonA*, RG192 F⁻ *lac ara-leu thi rif* (National Collection of Type Cultures, Colindale, London), UB5201 F⁻ *pro met nalA recA* (P. Bennett, Bristol University), AB2575 Hfr *ilv tsx thiA*, R4 Hfr *relA1 metB1*, and AB1621 F⁻ *ara gal tsx rpsL xyl mlt glpD thiA* (H. P. Charles, Reading University). Genotypic symbols follow Bachmann and Low (1980). Plasmids used were pWR23 *incIH2* Suc Lac Tc, pMIP233 *incIH3* Suc Tc, pFoLac *incFV/OF* Lac, pR821a *incI2* Pn, pRP4 *incP* Pn Km Tc, and pR6K *incX* Pn, Sm, harboured in *E. coli* K12 strains RG192, RG192, C600, J53-1,

HB101 and J53 respectively (Plasmid Reference Centre, Colindale). Plasmid nomenclature follows Novick *et al.* (1976).

Media and antibiotics

Complete medium (Alaeddinoglu and Charles, 1979) was used for maintenance and culture of all bacterial strains. Minimal medium (Vogel and Bonner, 1956) supplemented with glucose or other sugars 0.2% w/v was used for all selections in genetic crosses. Growth requirements were added to a final concentration of 40 µg/ml. Antibiotics (Sigma) were added to complete and minimal media at a final concentration of 40 µg/ml unless otherwise stated.

Sensitivity tests

A diffusion method with the multidisk technique (Sojka *et al.*, 1986) was used.

Genetic methods

Conjugative plasmid and chromosomal time-of-entry transfer was by filter and broth matings, respectively (Willets, 1984). Transduction with bacteriophage P1 was as described by Glansdorff (1965).

Plasmid isolation

Extraction methods of Birnboim and Doly (1979), Kado and Liu (1981) and Holmes and Quigley (1981), as described by Maniatis *et al.* (1982), were used. Agarose gel electrophoresis and visualisation of plasmid bands in agarose gels was as described by Maniatis *et al.* (1982). Plasmid sizes were estimated by comparing migration distances against reference plasmids of 142, 60.9, 34.6 and 6.1 kb extracted from strain 39R861 (Plasmid Reference Centre, Colindale).

Results

Antibiotic-resistance patterns in S. dublin

During 1985–86, 1099 *S. dublin* isolates (1047 from cattle, 32 from sheep, nine from other species, eight from feed and three from the environment) were screened for sensitivity to antibiotics (table I). Sensitivity to all antibiotics tested was shown by 22% of isolates; 44% were resistant to streptomycin, 12% to sulphonamides and 20% to both streptomycin and sulphonamides; 11 isolates (1%) were resistant to three or more antibiotics, and were designated multiresistant. This low percentage of multiresistance in *S. dublin* for the years 1985–86 reflects a continuing trend in Great Britain (Sojka and Hudson, 1976; Threlfall *et al.*, 1979; Sojka *et al.*, 1986).

Cell lysates of eight multiresistant *S. dublin* isolates were subjected to agarose-gel electrophoresis, to analyse plasmid content (fig. 1a, lanes A–E; fig. 1b, lanes A–E; fig. 2, lanes F and H). All harboured the 72.5-kb putative virulence plasmid (Terakado *et al.*, 1983; Chikama *et al.*, 1985; Helmuth *et al.*, 1985; Manning *et al.*, 1986). With one exception, all multiresistant isolates harboured at least one other plasmid. Isolate S4659/85, resistant to tetracycline, chloramphenicol, neomycin, sulphonamide, trimethoprim and streptomycin, harboured the 72.5-kb plasmid only (fig. 1b, lanes A, C, E) suggesting that resistance determinants were chromosomally integrated.

Resistance plasmid harboured by strain S4659/85

Mating experiments were set up to test whether promiscuous plasmids encoding antibiotic multires-

istance (Hardy, 1986) were transferable from strain S4659/85 to *E. coli* K12 recipient strains. In these tests selection was made for tetracycline, neomycin and chloramphenicol separately (table II). Significantly, transfer of resistance determinants was detected only after mating periods of 16 h or more and at a mating temperature of 30°C. Shorter mating periods or increased mating temperatures gave no growth on selection plates.

Dependence upon cell-to-cell contact for transfer of resistance determinants was tested by mixing cell-free supernates of strain S4659/85 before and after DNAase treatment with late log-phase cultures of *E. coli* K12 recipients. No recombinants were detected after mixtures which had been incubated for 16 h at 30°C were plated on selection media, suggesting that transfer was not the result of transduction by bacteriophage nor transformation by free DNA.

The frequency of transfer for each resistance determinant to *E. coli* recipient J62.1 was low (table II). Transconjugants selected for resistance to tetracycline were obtained at a frequency 100-fold lower than when selection was for chloramphenicol or neomycin resistances. It was possible that the concentration of tetracycline was close to the MIC, thus causing inhibition of growth of transconjugants. Transconjugants were tested for acquisition of the non-selected resistance determinants by replica plating (table II). Co-transfer was not always 100%, suggesting either F-like chromosomal transfer or plasmid breakdown.

To test for F-like transfer, mating experiments were made with a recombination-deficient mutant, UB5201*recA*, as recipient. Transfer and co-transfer frequencies (table II) were similar to mating with

Table I. Antibiotic resistance patterns of *S. dublin* isolates in 1985–86

Antibiotic resistance						Number of isolates	Percentage of isolates
None						247	22
	Sm					488	44
		Su				132	12
	Sm	Su				221	20
	Sm	Su	Tc			1	1
	Sm	Su	Tc	Nm	Pn	4	
	Sm	Su	Tc	Nm	Sxt	4	
		Su	Tc	Nm	Sxt	1	
			Tc		Pn	1	
						1099	

Antibiotics were: streptomycin (Sm) 10 µg disk, compound sulphonamide (Su) 50 µg, tetracycline (Tc) 10 µg, neomycin (Nm) 10 µg, ampicillin (Pn) 10 µg, sulphamethoxazole/trimethoprim (Sxt) 25 µg, chloramphenicol (Cm) 10 µg.

Fig. 1a. Cell lysates of five multiresistant *S. dublin* isolates run through 0.7% agarose and visualised by UV transillumination after ethidium bromide staining: S4909/85 Sm Su Tc Nm Pn Cm (lane A); S4809/85 Su Tc Nm Sxt Cm (lane B); S5135/85 Sm Su Tc Nm Pn Sxt Cm (lane C); S1445/85 Sm Su Tc Nm Sxt Cm (lane D); and S 4659/85 Sm Su Tc Nm Sxt Cm (lane E). Extraction was by the method of Birnboim and Doly (1979). Residual chromosomal DNA (chr) and the putative *S. dublin* virulence plasmid of 72.5-kb (vp) and its open circular form (oc) are indicated.

Fig. 1b. Cell lysates of multiresistant *S. dublin* isolates run through 0.7% agarose and visualised by UV transillumination after ethidium bromide staining: S4659/85 Sm Su Tc Nm Sxt Cm extracted by the methods of Kado and Liu (1981) (lane A), Birnboim and Doly (1979) (lane C), and Homles and Quigley (1981) as described by Maniatis *et al.* (1982) (lane E); S3717/85 Tc Pn Cm (lane B), S5260/85 Sm Su Tc Nm Sxt Cm (lane D) and sensitive strain S1447/85 (lane F) were extracted by the method of Birnboim and Doly (1979). Residual chromosomal DNA (chr) and the putative *S. dublin* 72.5-kb virulence plasmid (vp) are indicated.

recombination-proficient recipient J62.1. Independence from the recipient's recombination system indicated transfer by autonomous plasmid.

Physical evidence for plasmids in transconjugants

Repeated attempts to demonstrate a plasmid, other than the 72.5-kb plasmid, in strain S4659/85 failed and yet the evidence presented above indicated the involvement of a plasmid in determining resistance and its transfer. Transconjugant J62.1 and UB5201*recA* colonies picked at random directly from selection plates were analysed for plasmid content. Plasmids corresponding to about 87–94 kb were detected. Fig. 2 (lanes A–E) shows

Fig. 2. Cell lysates from five different UB5201*recA* transconjugants picked directly from tetracycline selection plates, after matings with strain S4659/85 as donor, were extracted by the method of Kado and Liu (1981) (lanes A–E). The same five UB5201*recA* transconjugants were also subcultured by streaking on selection plates containing tetracycline, and single colonies were extracted by the method of Kado and Liu (1981) (lanes I–M). Strains S5260/85 Sm Su Tc Nm Pn Cm (lane F) and S4659/85 (lane H) were extracted by the method of Kado and Liu (1981). Linear molecular weight markers, lambda *Hind*III digested DNA (lane G) were also run. Agarose concentration was 0.7%, and DNA species were visualised by UV transillumination after ethidium bromide staining. Residual chromosomal DNA (chr) is indicated.

the profiles of five UB5201*recA* transconjugants picked from selection plates containing tetracycline. Similar faint plasmid bands were demonstrated in both J62.1 and UB5201*recA* transconjugants picked from selection plates separately containing chloramphenicol and neomycin. Small differences in plasmid size were observed, but whether size variation was caused by deletion or was an artefact of the gels was unclear. Subcultured J62.1 and UB5201*recA* transconjugants did not harbour autonomous plasmids, suggesting either plasmid integration into the chromosome or plasmid suicide. Fig. 2 (lanes I–M) shows the profiles of the same five UB5201*recA* transconjugants exemplified in fig. 2 (lanes A–E) but after one subculturing on complete medium containing tetracycline.

Stable chromosomal integration of resistance determinants

If the plasmid was not surviving soon after transfer, loss of antibiotic resistance might be

Table II. Transfer of resistance determinants in mating experiments between *S. dublin* strain S4659/85 and *E. coli* K12 recipient strains

Recipient strain of <i>E. coli</i> K12	Marker selected	Frequency of transfer/donor	Percentage co-transfer of non-selected resistance determinant		
			Cm	Nm	Tc
J62.1	Cm	2×10^{-4}	...	100	80
	Nm	2×10^{-4}	100	...	80
	Tc	3×10^{-6}	74	74	...
UB5201 <i>recA</i>	Cm	3×10^{-4}	...	100	94
	Nm	4×10^{-4}	100	...	96
	Tc	7×10^{-5}	85	87	...

See footnote to table I; and see text for conditions of mating.

anticipated. To test this, about 20 transconjugant colonies picked from selection plates were tested for resistance patterns and then grown for about 50 generations in non-selective conditions. No differences in resistance pattern were detected, indicating very stable inheritance of resistance determinants.

An alternative explanation for loss of autonomous plasmid in transconjugants with stable inheritance of resistance determinants was that resistance determinants were encoded within transposable genetic elements. Upon suicide of the plasmid, survival of resistance determinants would require their transposition on to the chromosome. If this were so, transconjugants would not act as donors of resistance determinants in mating experiments, unless a helper plasmid was introduced to act as target and vector for transposable genetic elements. Plasmids pR821a, pRP4 and pR6K were

introduced into four J62.1 Tc^r Cm^r Nm^r transconjugants in separate experiments by filter mating (see *Methods*), selection being made for ampicillin resistance in each case. Ampicillin-resistant derivatives were then filter mated at 30°C for 16 h with strain AB1621 as recipient. Selection was made for ampicillin alone, to demonstrate helper plasmid transfer to strain AB1621, and for resistances not determined by the helper plasmid. For example, with pRP4 Pn Km Tc as helper, selection was made for chloramphenicol resistance. Transconjugants with and without helper plasmid did not donate resistance determinants at a detectable frequency in any mating experiment.

Mapping experiments were performed to demonstrate that the resistance determinants were chromosomally located in transconjugants. Bacteriophage P1 lysates were prepared from four J62.1 transconjugants chosen at random from different initial mating experiments. The transconjugants were resistant to tetracycline, chloramphenicol and neomycin (sulphonamide, trimethoprim and streptomycin were not tested). Lysates were used to transduce Hfr AB2575*ilv* and Hfr R4*met* to prototrophy and resistance. Frequencies of transduction (table III) were satisfactory. Co-transduction frequencies between resistance determinants showed that chloramphenicol and neomycin resistance determinants on the one hand, and the tetracycline resistance determinant on the other, were closely linked but separated by an intervening sequence homologous with the *E. coli* K12 chromosome permitting recombination.

Transductants of Hfr R4 and Hfr AB2575, resistant to the three antibiotics tested, were used as donors in time-of-entry conjugation experiments

Table III. Transduction of resistance determinants from transconjugants to *E. coli* K12 Hfr strains

Recipient	Marker selected	Frequency of transduction/donor phage	Percentage co-transduction of non-selected resistance determinants		
			Cm	Nm	Tc
Hfr AB2575 <i>ilv</i>	<i>ilv</i> ⁺	2.0×10^{-5}	0	0	0
	Cm	1.1×10^{-6}	...	100	68
	Nm	1.9×10^{-6}	100	...	66
	Tc	1.7×10^{-6}	73	73	...
Hfr R4 <i>met</i>	<i>met</i> ⁺	1.3×10^{-5}	0	0	0
	Cm	2.3×10^{-6}	...	100	72
	Nm	1.7×10^{-6}	100	...	71
	Tc	8.7×10^{-6}	69	69	...

See footnote to table I.

with *E. coli* K12 AB1621 as recipient. Hfr AB2575 donates its chromosome in an anticlockwise direction with a point of origin at minute 95 on the *E. coli* K12 linkage map. Hfr R4 donates clockwise from minute 5. The earliest time of entry of resistance determinants was 25 min with strain AB2575 and 85 min with strain R4, locating the resistance determinants at minute 20 of the *E. coli* K12 linkage map.

Chromosomally integrated plasmid of strain S4659/85 belonging to the incH2 incompatibility group

Plasmids of incompatibility group *incH2* are conjugally transmissible at 30°C or below (Johnson *et al.*, 1976), a feature in common with the chromosomally integrated plasmid in strain S4659/85. In reciprocal matings between strains S4659/85 and RG192 pWR23 (*incH2*), only rare microcolonies were detected on plates selecting for retention of both plasmids in one or other strain. Propagation of microcolonies by subculture on joint selection plates failed. In control experiments, to verify that strain S4659/85 could act as recipient as well as donor in such matings, plasmids pMIP233 (*incH3*) and pFolac (*incFV*) were shown to be compatible. That strain S4659/85 stably maintained *incH3* and *incFV* but not *incH2* plasmids suggests that an *incH2* plasmid was resident in the chromosome.

Discussion

The reason for the rarity of multiresistant *S. dublin* strains in Great Britain is a matter for speculation. It is possible that maintenance of plasmids, other than the 72.5-kb putative virulence plasmid, is selectively disadvantageous during the onset of infection. It seems unlikely that *S. dublin* is an inhospitable host to plasmids since plasmid-bearing strains were demonstrated and plasmids were readily introduced. Indeed, sensitive *S. dublin* strains have been shown to receive and maintain R plasmids representative of 15 incompatibility groups (unpublished data). Multiresistance plasmid absence from *S. dublin* remains intriguing.

Strain S4659/85 was unique in that it harboured a conjugally transmissible *incH2* plasmid in its chromosome. The extended mating period required for successful transfer and the possible size variation of plasmids in transconjugants suggested that

the plasmid in strain S4659/85 excised from the chromosome infrequently and possibly illegitimately. If the size variation of newly acquired plasmids in transconjugants was genuine and not an artefact of the gels, their inability to act as donors indicates loss of essential transfer functions. Whether that loss was caused by imprecise excision from the donor chromosome or as a result of recombination events upon entering *E. coli* is unknown.

Integration of the plasmid into the *E. coli* K12 chromosome of *UB5201recA* strongly suggests regions of homology between plasmid and chromosome and that the plasmid may encode its own recombination system, perhaps in the form of insertion sequences or transposons (Calos and Miller, 1980). Whether the chromosomal location of plasmid insertion in *E. coli* K12 is the same as that in *S. dublin* is unknown. Homology between plasmid and chromosome was confirmed by transductions in which crossovers between chromosome and intervening sequences within the antibiotic resistance encoding region were demonstrated.

If plasmids confer disadvantage upon *S. dublin*, by draining energy and metabolites from the central pool to support replication, for example, there exists a selective pressure upon plasmids to reduce that burden. Whether integration of plasmids into the chromosome alleviates that burden is debatable.

Helmuth and Seiler (1986) demonstrated in Southern hybridisations that a 700-bp internal DNA fragment, from the chloramphenicol acetyl transferase gene of pBR325, hybridised with chromosomal DNA from a plasmid-free multiresistant isolate of *S. dublin*. Although transfer of resistance determinants was not detected, and incompatibility and transposon tests were not done, it is possible that strain S4659/85 is the same as, or similar to, those isolates described by Helmuth and Seiler (1986). Furthermore, they went on to report a dramatic increase, from 16% to 70%, in this R plasmid-free type of *S. dublin* isolate in the Federal Republic of Germany in the mid-1970s.

Stability and conjugal transmissibility of the resistance phenotype of *S. dublin* S4659/85 is a cause for concern. If this isolate represents a virulent clone currently in circulation in Great Britain, we might see a dramatic increase in the isolation of multiresistant *S. dublin* from animal infections.

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