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Trimethoprim Resistance Conferred by W Plasmids in Enterobacteriaceae

By NAOMI DATTA AND R. W. HEDGES
Department of Bacteriology, Royal Postgraduate Medical School, Du Cane Road, London, W12 0HS

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SUMMARY

High-level resistance to trimethoprim (minimum inhibitory concentration > 1000 μg/ml) was conferred by R factors of the W compatibility group in *Escherichia coli* and *Klebsiella* spp. isolated from patients in three London hospitals. We suggest that we are observing the early stages in the spread of a new R factor.

INTRODUCTION

Trimethoprim, a synthetic inhibitor of folic acid metabolism in bacteria, has been used for the treatment of human infections in Western Europe for 3 to 4 years. The occurrence of trimethoprim-resistant enteric bacteria has been reported (Lebek & Weidmer, 1971; Fleming, Datta & Grüneberg, 1972; Lacey, Gillespie, Bruten & Lewis, 1972). Fleming *et al.* (1972) demonstrated that, in at least some cases, resistance was conferred by a transferable plasmid (R factor). We have tested trimethoprim-resistant strains of Enterobacteriaceae isolated in hospitals in the U.K. The levels of resistance varied considerably. High-level resistance (ability to grow on medium containing 1000 μg or more trimethoprim/ml) was always R factor determined. The determining plasmids all belonged to compatibility group W (Hedges & Datta, 1971).

The R^+ strains constituted a high proportion of trimethoprim-resistant cultures from three of seven hospitals. A majority of them were *Klebsiella* spp. with uniform and unusual biochemical characteristics. Cultures from the other four hospitals were resistant at lower levels and failed to transfer resistance. Most of these were klebsiellae with the biochemical characteristics typical of *Klebsiella aerogenes*.

METHODS

Trimethoprim-resistant cultures. Trimethoprim resistance was recognized in hospital diagnostic laboratories, usually by the use of discs containing 1·25 μg trimethoprim placed on cultures on suitable plates (Waterworth, 1969). Their characteristics are listed in Table 1. Each was isolated from a different patient.

Escherichia coli K12 *strains.* J53 *met pro* (Clowes & Hayes, 1968); J62 *pro his trp* (Clowes & Hayes, 1968); J62–1 *pro his trp nal-r* nalidixic acid-resistant mutant of J62; J62–2 *pro his trp rif-r* rifampicin-resistant mutant of J62; HfrC *met* (Clowes & Hayes, 1968).

Plasmids. R factors representative of the known compatability groups are listed in Table 2.

Phage. MS2 (Davis, Strauss & Sinsheimer, 1961).

Media. Minimal salts agar (Clowes & Hayes, 1968); nutrient broth no. 2 (Oxoid); MacConkey agar (Oxoid CM7b); diagnostic sensitivity test (DST) agar (Oxoid CM261).

Identification. Cultures were tested for production of indole and urease and ability to grow with citrate as carbon source. Strains which were indole +ve, urease −ve, citrate −ve and whose colonial morphology on MacConkey agar was typical of *Escherichia coli* were recorded as *E. coli*. All other strains were tested for motility; production of gas from glucose; fermentation of lactose, mannitol, dulcitol, sucrose, salicin and malonate; gelatine liquefaction; MR and VP (Barritt's method) reactions; and decarboxylation of arginine, ornithine and lysine. Methods were as described by Cowan & Steel (1965). For MR and VP tests cultures were incubated for 2 days at 37 °C. Tables from Cowan & Steel (1965) were used in naming species.

Determination of minimum inhibitory concentration (MIC). DST agar plates containing 4 % lysed horse blood and serial concentrations of trimethoprim were seeded with suspensions of bacteria at a dilution to give separate colonies on control plates. Plates were incubated overnight at 37 °C and the MIC recorded as that which prevented visible growth.

Transfer of R factors from wild strains to Escherichia coli K12. Overnight broth cultures of the wild strain and a K12 derivative (J62–1 if the wild strain was *nal-s*, J62–2 if the wild strain was *nal-r*) were mixed in a ratio of one part donor to nine parts recipient and incubated overnight at 37 °C. The mixture was plated on minimal salts agar with appropriate amino acids, nalidixic acid, 25 μg/ml (or rifampicin, 50 μg/ml) and trimethoprim, 5 μg/ml, and the plates were incubated for 24 h at 37 °C. Colonies appearing on these plates were streaked on DST plates containing 4 % lysed horse blood and trimethoprim, 5 μg/ml. Well isolated colonies with appearance typical of *E. coli* K12 were identified by their nutritional requirement and their sensitivity pattern was tested. Clones satisfying all tests were taken to be R^+ derivatives of J62–1 (or J62–2).

Transfer of R factors between lines of Escherichia coli K12. As described in Coetzee, Datta & Hedges (1972).

Transfer of the R factor S-a to Klebsiella strains. When J53 (S-a) was used as donor to Klebsiella strains the procedure was that used for matings between strains of *Escherichia coli* K12 except that mating mixtures were incubated overnight and plated on minimal medium with sodium citrate (0·2 %, w/v) as carbon source and 7 μg kanamycin/ml of medium.

Determination of fi character. As described in Datta *et al.* (1971).

Tests for compatability. As described in Coetzee *et al.* (1972).

RESULTS

Minimal inhibitory concentrations of trimethoprim. The MICs are listed in Table 1.

Transfer of trimethoprim resistance. Trimethoprim (Tp) resistance was transferred to *Escherichia coli* K12 (J62–1 or J62–2) from 19 strains, always accompanied by sulphonamide (Su) resistance. In many cases resistance to other drugs was also transferred, although selection was for Tp resistance only (Table 1).

Transfer between lines of Escherichia coli K12. The Tp-resistant J62–2 strains were used as donors in matings with J53 and HfrC, selection being for Tp-resistance. In 13 cases Su- and Tp-resistance was transferred without resistance to other drugs. The HfrC R+ derivatives of these 13 were visibly lysed by phage MS2 (i.e. the R factors were fi^-). These were R388, R405, R406, R407, R408, R411, R413, R419, R420, R421, R422, R423, R424. In the other six cases, resistance to antibiotics (one or more of the following: ampicillin, streptomycin, tetracycline, chloramphenicol) was always transferred with resistance to Su and Tp. None of the HfrC R+ derivatives of these crosses were visibly lysed by MS2 (i.e. fi^+ R factors were

Table 1. *Naturally occurring trimethoprim-resistant bacteria*

No.	Source*	Species	Resistance pattern†	MIC Tp (μg/ml)	Resistance transferred to J62-1 or J62-2‡ (selection for Tp resistance)	R factor no.
D769§	P	*Escherichia coli*	SuTp	> 1000	SuTp	R388
D798	P	*E. coli*	TSuTp	> 1000	TSuTp	R409
D800	P	*E. coli*	SuTp	> 1000	SuTp	R411
D795	P	*Klebsiella aerogenes*	ATCSuTpNal	> 1000	SuTp	R406
D770‖	P	*Klebsiella* sp¶	ASTCSuTp	> 1000	STCSuTp	R389
D792	P	*Klebsiella* sp	ASTCSuTp	> 1000	STCSuTp	R403
D793	P	*Klebsiella* sp	ASTCSuTpNal	> 1000	ASTCSuTp	R404
D794	P	*Klebsiella* sp	ASTCSuTp	> 1000	ASTCSuTp	R405
D796	P	*Klebsiella* sp	ASTCSuTp	> 1000	SuTp	R407
D797	P	*Klebsiella* sp	ASCSuTp	> 1000	SuTp	R408
D799	P	*Klebsiella* sp	ASTCSuTpNal	> 1000	STCSuTp	R410
D801	U	*Klebsiella* sp	ASTCSuTp	> 1000	ASTCSuTp	R412
D802	U	*Klebsiella* sp	ASTCSuTpNal	> 1000	ASTCSuTp	R413
D805	U	*Klebsiella* sp	ASCSuTpFuNal	> 1000	SuTp	R419
D806	U	*Klebsiella* sp	ASTCSuTp	> 1000	ASTCSuTp	R420
D808	W	*Klebsiella* sp	ASTCSuTp	> 1000	SuTp	R421
D809	W	*Klebsiella* sp	ASTCSuTpFuNal	> 1000	ASTCSuTp	R422
D810	W	*Klebsiella* sp	ASTCSuTp	> 1000	ASTCSuTp	R423
D811	W	*Klebsiella* sp	ASTCSuTp	> 1000	ASTCSuTp	R424
D829	U	*Klebsiella* sp	ASCSuTp	> 1000	—	—
D830	U	*Klebsiella* sp	ASSuTp	> 1000	—	—
D831	P	*E. coli*	SuTp	64	—	—
D832	P	*E. coli*	SuTp	32	—	—
D833	P	*E. coli*	SuTp	256	—	—
D834	U	*E. coli*	STSuTp	64	—	—
D837	P	*E. coli*	ASSuTp	128	—	—
D845	H	*E. coli*	ASTKSuTpFuNal	16	—	—
D852	B	*E. coli*	ASKSuTpNal	32	—	—
D855	B	*E. coli*	ASKSuTp	32	—	—
D865	G	*E. coli*	ASTCSuTp	32	—	—
D847	M	*K. pneumoniae*	ASTCKSuTpFuNal	128	—	—
D835	U	*K. aerogenes*	ASSuTp	128	—	—
D836	U	*K. aerogenes*	ACSuNal	32	—	—
D807	U	*K. aerogenes*	ASTSuTpFu	128	—	—
D838	H	*K. aerogenes*	ASCSuTpFuNal	64	—	—
D839	H	*K. aerogenes*	ASTCKSuTpFu	64	—	—
D840	H	*K. aerogenes*	ASTCSuTpFuNal	256	—	—
D841	H	*K. aerogenes*	ASTCKSuTp	128	—	—
D842	H	*K. aerogenes*	ATpNal	128	—	—
D843	H	*K. aerogenes*	ASTCKSuTpNal	256	—	—
D844	H	*K. aerogenes*	ASTCKSuTpNal	128	—	—
D846	H	*K. aerogenes*	ASTCKSuTpFuNal	128	—	—
D848	M	*K. aerogenes*	ASTCSuTp	32	—	—
D849	M	*K. aerogenes*	ASTCKSuTpFuNal	128	—	—
D850	B	*K. aerogenes*	ACTpFuNal	16	—	—
D851	B	*K. aerogenes*	ASSuTp	8	—	—
D853	B	*K. aerogenes*	ASCSuTpFuNal	8	—	—
D854	B	*K. aerogenes*	ACTpFuNal	32	—	—
D861	G	*K. aerogenes*	ASCKSuTpNal	256	—	—
D862	G	*K. aerogenes*	ASTCKSuTpNal	128	—	—
D864	G	*K. aerogenes*	ASTCSuTpNal	256	—	—

* P, St Pancras Branch, University College Hospital, London; U, University College Hospital, Gower St, London; W, Whittington Hospital, Highgate, London; H, Hammersmith Hospital, London; M, West Middlesex Hospital, London; B, Royal Infirmary, Bristol; G, Royal Infirmary, Glasgow.

† Symbols for resistance to: ampicillin (A), streptomycin (S), tetracycline (T), chloramphenicol (C), kanamycin (K), sulphonamides (Su), trimethoprim (Tp), furazolidone (Fu), nalidixic acid (Nal).

‡ Single clone, selected on minimal salts agar supplemented with proline, histidine, tryptophan, trimethoprim and either nalidixic acid or rifampicin.

§ D769 was strain EC1 (Fleming *et al.* 1972). It was distinguishable from D798 and D800 (see text).

‖ D770 was strain KA1 (Fleming *et al.* 1972).

¶ Strains listed as *Klebsiella* sp. were MR+ VP+ malonate-ve (see text).

present). R389 was further studied as an example of these. (R389 was the R factor reported as *fi*+ by Fleming *et al.* 1972.)

When J62–1(R389) was used as donor, and selection was made for Tp resistance, every clone tested (50/50) was resistant to streptomycin (S), tetracycline (T) and chloramphenicol (C), as well as to Su and Tp even though mating was interrupted after 5 min. When chloramphenicol was used for selection, resistant recipients had either of two resistance patterns, STCSu or STCSuTp. HfrC with either pattern showed no visible lysis by phage MS2, thus the *fi*+ character was always associated with resistance to STCSu.

Compatibility of Tp-resistance plasmids. The first R factor conferring Tp-resistance to be identified, R388, was transferred from J62–1 to J53 and J53 carrying plasmids of diverse compatibility groups. No exclusion of R388 by any resident plasmid was observed. However, R factor S-a, a W plasmid, was eliminated from all R388+ clones tested (20/20). R388 co-existed stably with all the other R factors. The frequency of transfer of R388 was reduced by the presence of RP4 when both factors were present in the donor culture (Table 2).

R factor S-a was introduced into lines of J62–1 or J62–2 carrying each of the other transmissible Tp-resistance plasmids R389, R403, R404, R405, R406, R407, R408, R409, R410, R411, R412, R413, R419, R420, R421, R422, R423 and R424. In no case did the resident plasmid exclude transfer of S-a but in every case resistance to trimethoprim was lost. With R389 resistance to tetracycline was retained (the continued presence of the streptomycin, chloramphenicol and sulphonamide resistances of R389 could not be detected in the presence of S-a). S-a was introduced into naturally occurring Klebsiella strain D829 (Table 1) which was highly resistant to Tp (but did not transfer resistance) and Tp resistance was eliminated. We were unable to detect transfer of S-a to D830.

Distribution of R factors determining Tp-resistance. Of the 20 wild strains in which Tp resistance was determined by a W plasmid, 17 were *Klebsiella* spp. and three were *Escherichia coli.* Of the 17 Klebsiella strains one, D795, was typical *Klebsiella aerogenes* (Cowan & Steele, 1965) the other 16 were all MR+ VP+ malonate −ve i.e. could not be classified as either *K. aerogenes* or *K. pneumoniae.* D830 was similar and was resistant to > 1000 μg trimethoprim/ml, but we have no direct evidence that its Tp resistance was plasmid-determined. Of the 3 *E. coli*, one (D769) belonged to O group 18 (Fleming *et al.* 1972): the other two could not be grouped with a limited range of antisera, but were not O18, and differed from one another biochemically. D798 fermented dulcitol, D800 did not (Table 1). The one *K. aerogenes* and the three *E. coli* all came from one hospital (University College Hospital, St. Pancras Branch). All the strains carrying Tp resistance plasmids were isolated from patients at University College Hospital (Gower St. and St Pancras branches, which are over a mile apart) and the Whittington Hospital, Highgate.

Strains of bacteria resistant to trimethoprim but with MICs < 1000 μg/ml are listed in Table 1. There were nine isolates of *Escherichia coli*, 20 of typical *Klebsiella aerogenes*, and one of *K. pneumoniae.* None transferred Tp resistance. They were isolated at University College Hospital, Hammersmith Hospital, the West Middlesex Hospital, Bristol Royal Infirmary and the Royal Infirmary, Glasgow.

DISCUSSION

In all naturally occurring strains of bacteria resistant to 1000 μg trimethoprim/ml medium, the resistance was determined by a plasmid of the W compatibility group. Only with D830 have we no direct evidence for this statement. Its Tp resistance was non-transmissible and the strain failed to accept S-a, so elimination by a W plasmid could not be tested.

Table 2. *Compatibility of R388 with R factors of various groups*

Donor	Recipient	Compatibility group of resident R factor	Reference	Frequency of transfer	Exconjugant clones tested for presence of each plasmid	Transfer frequency from 'doubles': selection for transfer of: R388	Other
J62(R388)	J53	—	—	3×10^{-3}	—	—	—
	J53(R1)	FII	Hedges & Datta (1972)	1×10^{-2}	20/20 both present	3×10^{-3}	9×10^{-3}
	J53(R124)	FIV	Hedges & Datta (1972)	7×10^{-3}	20/20 both present	1×10^{-2}	8×10^{-3}
	J53(R144)	I	Lawn, Meynell, Meynell & Datta (1967)	9×10^{-3}	20/20 both present	2×10^{-2}	2×10^{-2}
	J53(R46)	N	Datta & Hedges (1971)	1×10^{-2}	20/20 both present	5×10^{-3}	6×10^{-3}
	J53(S-a)	W	Hedges & Datta (1971)	1×10^{-3}	20/20 R388 only	—	—
	J53(RP4)	P	Datta *et al.* (1971)	1×10^{-3}	20/20 both present	2×10^{-5}	3×10^{-4}
	J53(R391)	J	Coetzee *et al.* (1972)	1×10^{-3}	20/20 both present	2×10^{-2}	7×10^{-6}
	J53(RA1)	A	Hedges & Datta (1971)	1×10^{-3}	20/20 both present	5×10^{-3}	3×10^{-3}
	J53(R300)	—	Lawn *et al.* (1967)	5×10^{-3}	20/20 both present	3×10^{-2}	1×10^{-4}

Mating mixtures were incubated for 1 h. Dilutions were plated on minimal medium supplemented with appropriate nutrients and antibacterial drugs. Trimethoprim was used at 10 μg/ml medium. Frequencies of transfer were calculated per donor cell. From each 'double' R388 was transferred independently of the other plasmid.

The only previously identified R factors of the W group did not confer resistance to trimethoprim. They were derived from strains of *Shigella* and *Aeromonas* from Japan (Hedges & Datta, 1971) and of *Proteus rettgeri* from Greece (Kontomichalou, 1971; Coetzee *et al.* 1972). Like the ones described here they showed mutual incompatibility but no mutual surface exclusion. Most of the Tp-resistance plasmids were self-transmissible and conferred resistance to Su and Tp only. One Tp plasmid (in D829) was not transmissible but was shown to be a W plasmid by elimination by R factor S-a. We think it likely that the Tp resistance of D830 was of the same nature. In some cases, e.g. D770, resistance to Tp and Su was transferred only in company with resistance to other drugs. From the K12 recipients, Tp resistance could be eliminated by introduction of S-a. We believe the original bacteria were 'doubles', carrying non-self-transmissible W plasmids conferring resistance to Tp and Su and *fi*+ R factors, bearing the other resistance markers. Transmissibility was the only difference observed among the Tp plasmids which suggests that they were all produced by replication of a single original replicon. This was also suggested by their geographical distribution which was confined to three London hospitals, separated from one another by several miles. Between two of them (the two branches of University College Hospital) there is some interchange of patients and staff but with the third (the Whittington) exchange is limited to annual rotation of some junior medical staff.

The hosts for the R factors were Enterobacteriaceae, isolated from infected patients. Most of them were members of the genus *Klebsiella* with unusual biochemical characteristics, not conforming with the definitions of any Klebsiella species (Cowan & Steele, 1965). All but one of the R+ Klebsiella strains were closely similar biochemically, although their resistance patterns varied, and it seems likely that they were members of a single clone present in these hospitals in the Camden area of London. The R factors had been transferred to one strain of typical *Klebsiella aerogenes* and to three distinguishable strains of *Escherichia coli*. The spread of the predominant host and the spread of the R factor to other bacteria demonstrates the two mechanisms by which an R factor may be distributed in nature. There is a parallel between the dissemination of trimethoprim resistance, determined by W plasmids, and that of carbenicillin resistance, mediated by P plasmids in *Pseudomonas aeruginosa* and *Proteus* and *Klebsiella* spp. in the MRC Industrial Injuries and Burns Unit in Birmingham (Datta *et al.* 1971; Roe & Lowbury, 1972). In each case the R factor belonged to a compatibility group seldom or never described before; thus apparently previously rare types of plasmid are spreading epidemically. The probable selective advantage enjoyed by the P plasmids in the Burns Unit was their ability to infect *P. aeruginosa*, conferring resistance to carbenicillin. For the W plasmids it is that they confer resistance to a new drug trimethoprim.

Since trimethoprim is widely used, and not especially in the three hospitals where the R factors were found, we anticipate a dissemination of the R factor to other hospitals and through bacterial populations generally.

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REFERENCES

CLOWES, R. C. & HAYES, W. (1968). *Experiments on Microbial Genetics.* Oxford & Edinburgh: Blackwell Scientific Publications.

COETZEE, J. N., DATTA, N. & HEDGES, R. W. (1972). R factors from *Proteus rettgeri. Journal of General Microbiology* **72** (in the press).

COWAN, S. T. & STEEL, K. J. (1965). *Manual for the Identification of Medical Bacteria.* Cambridge: University Press.

DATTA, N. & HEDGES, R. W. (1971). Compatibility groups among *fi*⁻ R factors. *Nature, London* **234**, 222–223.

DATTA, N., HEDGES, R. W., SHAW, E. J., SYKES, R. & RICHMOND, M. H. (1971). Properties of an R factor from *Pseudomonas aeruginosa. Journal of Bacteriology* **108**, 1244–1249.

DAVIS, J. E., STRAUSS, J. H. & SINSHEIMER, R. L. (1961). Bacteriophage MS2: another RNA phage. *Science, New York* **134**, 1427.

FLEMING, M. P., DATTA, N. & GRÜNEBERG, R. N. (1972). Trimethoprim resistance determined by R factors. *British Medical Journal* **1**, 726–728.

HEDGES, R. W. & DATTA, N. (1971). *fi*⁻ R factors conferring chloramphenicol resistance. *Nature, London* **234**, 220–221.

HEDGES, R. W. & DATTA, N. (1972). R124, an *fi*⁺ R factor of a new compatibility class. *Journal of General Microbiology* **71**, 403–405.

KONTOMICHALOU, P. (1971). *R factors controlling resistance to the penicillins.* Habilitation Thesis, University of Athens.

LACEY, R. W., GILLESPIE, W. A., BRUTEN, D. M. & LEWIS, E. (1972). Trimethoprim resistant coliforms. *Lancet* **i**, 409–410.

LAWN, A. M., MEYNELL, E., MEYNELL, G. G. & DATTA, N. (1967). Sex pili and the classification of sex factors in the *Enterobacteriaceae. Nature, London* **216**, 343–346.

LEBEK, G. & WIEDMER, E. (1971). Empfindlichkeit menschlicher Krankheitserreger gegen das Kombinations-Therapeutikum Sulfamethoxazol-Trimethoprim in vitro. *Schweizerische Medizinische Wochenschrift* **101**, 1385–1390.

ROE, E. & LOWBURY, E. J. L. (1972). Changes in antibiotic sensitivity patterns of Gram-negative bacilli in burns. *Journal of Clinical Pathology* **25**, 176–178.

WATERWORTH, P. M. (1969). Practical aspects of testing sensitivity to trimethoprim and sulphonamide. *Postgraduate Medical Journal* (Suppl) **45**, 21–27.