Restriction of DNA in *Yersinia enterocolitica* Detected by Recipient Ability for a Derepressed R Factor from *Escherichia coli*

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SUMMARY

A derepressed R factor, RY2drd2, was transferred at a frequency of 5×10^{-3} between two strains of *Yersinia enterocolitica* mated on a membrane. Under the same conditions transfer of this R factor from *Escherichia coli* to *Y. enterocolitica* was observed at a frequency of only 7.7×10^{-6} . This frequency was greatly increased when the recipient strain was heat-treated before mating. Heat exposure for optimum fertility was 50 to 52 °C for a period of 2 to 3 min. Mutants of *Y. enterocolitica* were isolated which were infected by RY2drd2 from *E. coli* or from *Y. enterocolitica* at the same frequency. These observations strongly suggest that a DNA restriction and modification system in *Y. enterocolitica* causes its low recipient ability for plasmids from other species.

INTRODUCTION

The first report of the transfer of an F episome to a species in the genus *Yersinia* was that of *Flac*⁺ to *Y. pestis* (Martin & Jacob, 1962). Lawton, Morris & Burrows (1968) observed *Flac*⁺-mediated transfer of chromosome between auxotrophic strains of *Y. pseudotuberculosis*. Thus chromosomal mapping in this species was initiated (Morris & Burrows, 1968; Lawton & Stull, 1971), and developed by the use of an Hfr clone (McMahon, 1971, 1973).

Chromosomal transfer mediated by an FCM was also reported in *Y. pestis* (Lawton & Stull, 1972). However, the same authors found no chromosomal transfer mediated by *Flac*⁺ or FCM plasmid in *Y. enterocolitica*.

The recipient ability of *Y. pestis* and *Y. pseudotuberculosis* for R factors was demonstrated soon after the discovery of these plasmids (Ginoza & Matney, 1963). Transfer of R factors to *Y. enterocolitica* was obtained by Knapp & Lebek (1967) and by Rusu, Baron & Lazaroae (1970). Recently, strains of *Y. enterocolitica* carrying R factors have been found in nature (Cornelis, Wauters & Bruynoghe, 1973a; Cornelis, Wauters & Vanderhaeghe, 1973b). However, R factor-carrying *Y. enterocolitica* isolates are rare and experimental transfer of F or R factors from *Escherichia coli* or *Shigella flexneri* to species of *Yersinia* occurs at low frequency.

We have considered the possibility that this low frequency resulted from the degradation of foreign DNA by restriction and modification (R-M) systems. These R-M systems consist of two enzymes. One, the restriction endonuclease, causes double strand breaks in invading DNA whereas the other, the modification methylase, protects the DNA of the bacterial cell itself by methylation of a base on both strands of the nucleotide sequence specific for the

Table 1. *Bacterial strains*

Species	Strain number	Serotype	Relevant genotype	Origin
<i>Escherichia coli</i> K12	Richter		Defective Hfr	E. Meynell
	84Nx		F ⁻ <i>met cys try lac nal</i>	P. Frédéricq
<i>Yersinia enterocolitica</i>	W222	O:3	Wild type	From man
	W230	O:3	Wild type	From pig
	W227 }	O:9	Wild type	From man
	W277 }			
	W22701	O:9	<i>str</i>	From W227
	W22702, W22705 }	O:9	—	From W227*
	W22707, W22709 }			
	W22708	O:9	<i>str</i>	From W22703

* RY2*drd2* infected after NTG mutagenesis, then cured.

endonuclease (review: Meselson, Yuan & Heywood, 1972). R-M systems were first discovered and extensively studied in *E. coli* (review: Atber & Linn, 1969), but have been detected in many Gram-negative bacilli, including *Salmonella typhimurium* (Colson & Colson, 1967), *Shigella flexneri* (Falkow & Formal, 1969), *Citrobacter freundii* (de Graaf & Stouthamer, 1971) and *Pseudomonas aeruginosa* (Rolfe & Holloway, 1969).

We report on the recipient capacity of four strains of *Y. enterocolitica* for plasmid DNA from *E. coli* and present evidence which indicates that these strains exercise DNA restriction.

METHODS

Bacterial strains. All the strains are listed in Table 1. The *Y. enterocolitica* strains were from the collection of Professor G. Wauters, Department of Microbiology, University of Louvain. These strains were of serotype 3 or 9 (Winblad, 1967; Wauters, Le Minor & Chalon, 1971). These serotypes belong to two different biotypes (Nilhen, 1969; Wauters, 1970) and are those to which most strains encountered in man belong.

Plasmids. An *Flac*⁺ episome from strain 94 of R. C. Clowes was received from Professor P. Frédéricq. The R factor RY2*drd2* is a derepressed mutant of RY2 (fi⁺ Km Cm Sm Su) found in the strain *Y. enterocolitica* 857 (Cornelis *et al.* 1973*a*). This mutant was obtained by the method of Meynell & Datta (1967). Wild-type RY2 was introduced into strain Richter which was then treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Fluka) (100 µg/ml, pH 6 for 25 min). Plates bearing semi-confluent colonies were replicated to lawns of 84Nx (*met cys try lac*⁺) on plates of citrate Simmons agar as minimal medium (Frédéricq & Delhalle, 1972) supplemented with lactose, cysteine and tryptophan. Out of 16 clones of 84Nx growing in the absence of methionine and containing RY2, two were lysed by the male specific phages f1 and f2.

Media. The complete media were nutrient broth (Difco), MacConkey agar (Difco) and brain-heart agar (Difco) supplemented with 10% blood. The minimal medium for *Y. enterocolitica* was that described by Burrows & Gillett (1966) supplemented with 5 µg thiamine/ml. All incubations of *Y. enterocolitica* were performed at 28 °C.

Mutagenesis. Cultures of *Y. enterocolitica* were mutagenized by a 15 min treatment with 100 µg NTG/ml in 0.067 M-phosphate buffer at pH 6. The bacteria were washed twice, diluted in broth and incubated overnight.

Heat-treatment of the recipient followed by mating in liquid. A three ml sample of an 18 h culture in broth of the recipient strain was centrifuged and the bacteria were resuspended in

0.05 ml saline pre-heated to the chosen temperature. At fixed times, the cell suspensions were transferred to an ice bath, allowed to cool and centrifuged. The pellet of bacteria was immediately resuspended in 0.5 ml of an exponential culture of the donor and the mixtures were kept at 37 °C for 60 or 90 min.

Mating on membranes. An overnight culture of the donor strain was diluted 1:10 in fresh broth and incubated for 2½ h at 37 °C. Three ml of this culture were mixed with 5 ml of an overnight culture of the recipient strain and the mixture was filtered on a 25 mm Millipore membrane. The membrane was placed on the surface of a plate of brain-heart agar supplemented with blood and incubated for 2 h at 37 °C. The membrane was then removed and the bacteria were resuspended in 2 ml saline, diluted and plated. The frequencies of transfer were calculated in respect of donor input.

Selections. The bacteria carrying RY2drd2 were selected on 16 µg chloramphenicol/ml; *Y. enterocolitica* were selected on 35 µg carbenicillin/ml (Cornelis *et al.* 1973b); *E. coli* 84NX was selected on 16 µg nalidixic acid/ml; w22701 and w22708 were selected on 1000 µg streptomycin/ml.

Curing. Spontaneous R⁻ segregants of *Y. enterocolitica* were obtained by subculturing the corresponding R⁺ strains (Cornelis *et al.* 1973a).

RESULTS

Effect of pre-heating on the recipient ability of Y. enterocolitica

It was observed in preliminary experiments that pre-heating cultures of *Y. enterocolitica* at 50 °C before mating in liquid with *E. coli* donors allowed the recovery of Flac⁺- or RY2drd2-infected *Y. enterocolitica* clones at a significantly higher frequency than without heat treatment.

Two types of experiments were performed to determine the conditions of heat-treatment for optimal transfer of RY2drd2 from *E. coli* to *Y. enterocolitica* w227. In the first set of experiments, a culture of the recipient bacteria was divided into 3 ml samples that were exposed for 1 min to temperatures from 42 to 60 °C as described in Methods. Each treated sample was mixed with 0.5 ml of a single culture of the donor. The results of five independent experiments are summarized in Fig. 1. Although the absolute optimum frequency of transfer varied widely among experiments (from 1×10^{-7} to 5×10^{-6}) the optimum was always within the range 50 to 52 °C. Transfer was never observed (under these conditions) to recipient cells pre-heated at temperatures lower than 46 °C or higher than 54 °C, or to untreated controls.

In the second set of experiments, samples of the recipient cultures were exposed to the optimal temperature (51 °C) for various periods. The results of six independent experiments are summarized in Fig. 2. Again the absolute value of the optimum frequency varied between experiments (from 1×10^{-6} to 5×10^{-5}) but an optimum time of exposure was found within the range 2 to 3 min.

This heat effect on fertility was not confined to the transfer of RY2drd2 to strain w227 only. Table 2 shows that three other *Y. enterocolitica* strains exhibited increased recipient ability for RY2drd2 after exposure to 51 °C for 3 min, and that heat-treatment of w227 increased its recipient ability for an Flac⁺ factor.

Isolation and properties of Y. enterocolitica mutants with an increased recipient ability

The experiments in the previous section showed that the low recipient ability of *Y. enterocolitica* is determined in a positive way by a cellular factor having the same thermolability

Fig. 1

Fig. 2

Fig. 1. Optimum heating temperature for the transfer of RY2drd2 from *E. coli* 84NX to *Y. enterocolitica* w227. The heat treatments and matings were done as described in Methods. In each experiment, the highest frequency of transfer occurred in the 50 to 52 °C range and this frequency was taken as 100 %. Frequencies from the other temperature ranges were expressed as percentages of that at the optimum temperature. The results of five independent experiments were averaged. Bars represent the variation between experiments.

Fig. 2. Optimum time of heating at 51 °C for the transfer of RY2drd2 from *E. coli* 84NX to *Y. enterocolitica* w227. The heat treatments and matings were performed as described in Methods. The highest number of transfers was observed at 3 min in four experiments and at 1 and 2 min in two other experiments. For each experiment, the frequency of transfer at the optimum heating time was taken as 100 %. Frequencies from other heating times were expressed as percentages of that at the optimum time. The results of six independent experiments were averaged. Bars represent the variation between experiments.

as that which is characteristic of the restriction enzyme in several R-M systems (Arber & Linn, 1969). This suggested that restriction-deficient mutants of *Y. enterocolitica* with increased recipient ability could be isolated.

NTG mutagenized cultures of w227 were mated for 1 h in broth with strain 84NX and the transfer of RY2drd2 was selected. Under these conditions of mating, transfer of the R factor to wild-type w227 was not observed (see Table 2). Among five crosses with mutagenized cultures, two yielded respectively 1 and 31 RY2drd2-infected clones. Six of these clones were subsequently cured of the R factor and their recipient ability for RY2drd2 from *E. coli* was then compared with that of w227; this was done in membrane mating experiments rather than broth mating, to improve the overall frequency of transfer (McMahon, 1971). It was found that five of these six clones showed a more than 100-fold greater recipient ability than w227 (Table 3).

For most R-M systems in which restriction-deficient mutants were isolated, roughly half such mutants were found to be simultaneously deficient in the restriction and the modification functions. Therefore a series of crosses was performed, using each of these mutants as donor and a pair of streptomycin-resistant recipient strains. One derived from w227 (presumed restriction proficient, r^+) and the other from the mutant w22703 (presumed restriction deficient, r^-) (Table 4). Control crosses between wild-type strains showed that, once

Table 2. *Effect of heating on the transfer of RY2drd2 and Flac⁺ from E. coli to Y. enterocolitica*

Cultures of the recipient strains were heat-treated at 51 °C for 3 to 5 min, then mated for 1 h in liquid.

Plasmid	Recipient strain	Number of transfers per 5×10^6 donors	
		Unheated recipient strain	Heated recipient strain
RY2drd2	W222	1.3	18
	W222	0.3	1.5
	W230	48	439
	W227	0	24
	W227	0	380
	W227	0	500
	W277	0	2000
	W277	0	80
	W277	0	3
Flac ⁺	W277	6.3	37
	W277	1.5	500
	W277	2.7	117

Table 3. *Frequencies of transfer of RY2drd2 from E. coli 84Nx to wild type and mutants of Y. enterocolitica with increased recipient ability*

Recipient strains	Frequencies of transfer	Recipient strains	Frequencies of transfer
W227	7.7×10^{-6}	W22707	8.1×10^{-6}
W22702	1.4×10^{-2}	W22709	1.3×10^{-3}
W22703	5.6×10^{-3}	W22701	2.7×10^{-5}
W22704	9.3×10^{-3}	W22708	6.3×10^{-3}
W22705	1.5×10^{-2}		

Table 4. *Frequencies of transfer of RY2drd2 between wild type and mutants of Y. enterocolitica with increased recipient ability*

Donor strain	Frequency of transfer to recipient strain:	
	W22701	W22708
W227	5.0×10^{-3}	1.0×10^{-2}
W22702	1.3×10^{-2}	5.0×10^{-2}
W22703	1.2×10^{-2}	2.8×10^{-2}
W22704	4.8×10^{-3}	6.1×10^{-3}
W22705	5.8×10^{-3}	1.2×10^{-2}
W22707	2.4×10^{-2}	5.4×10^{-2}
W22709	2.5×10^{-3}	4.0×10^{-3}

established in *Y. enterocolitica*, RY2drd2 is transferred at the higher frequency, comparable to that observed when it is transferred from *E. coli* to the presumed r^- mutants. In the crosses with the mutant donors, the transfer of the R factor was observed at the high frequency to both the presumed r^+ and r^- recipient strains. Thus all five mutants, representing at least two independent mutations, have the r^-m^+ phenotype.

DISCUSSION

Most R-M systems in bacteria have been first detected by their effect on the efficiency of plating of bacteriophages. However, it is well known that bacterial and plasmid DNA can also be affected by R-M systems. A lowered apparent frequency of transfer and of recombination is observed when recipient cells exercise a restriction against the donor-type DNA (Arber & Morse, 1965).

Some R-M systems have been detected first from their effect on the recipient ability for plasmids and bacterial chromosome. Mutants with increased recipient ability were isolated and, in most cases, proven to be restriction-deficient, by measuring the efficiencies of plating of suitable bacteriophages (Colson & Colson, 1967; Okada, Watanabe & Miyake, 1968; de Graaf & Stouthamer, 1971).

The mutants isolated in this study have not yet been characterized further with bacteriophages. All of them had the $r^{-}m^{+}$ phenotype, in respect of their recipient and donor ability for RY2drd2. Thus these strains could not be used to produce phage lysates lacking the *Y. enterocolitica* modification in strains virtually isogenic to that with the wild-type ($r^{+}m^{+}$) phenotype.

Further characterization of the R-M system(s) in our strains of *Y. enterocolitica* can be done in two ways. One is to isolate a phage with a broad host range, so that a 'natural host' lacking the modification would be available. The other is to isolate more fertile mutants, assuming that the absence of $r^{-}m^{-}$ mutants in this study results from the small number of mutants obtained. However, some R-M systems are known in which all the mutations obtained had the $r^{-}m^{+}$ phenotype (Yoshimori, Roulland-Dussoix & Boyer, 1972).

In addition to the isolation of fertile mutants, the results of this work suggest the existence of an R-M system in *Y. enterocolitica* in two ways. First, the frequency of transfer of RY2drd2 between strains of *Y. enterocolitica* is the same as that from *E. coli* to the r^{-} mutants, and 10^3 times higher than from *E. coli* to *Y. enterocolitica*. Second, the conditions of heat-treatment for optimal transfer of plasmids from *E. coli* to *Y. enterocolitica* have been found to be closely similar to those which have been used to overcome phage restriction in *Salmonella* (Uetake, Toyama & Hagiwara, 1964; Mojica-a & Middleton, 1971), in *E. coli* (Lederberg, 1965; Schell & Glover, 1966) and *Citrobacter freundii* (de Graaf, Kreuning & van de Putte, 1973).

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