Host Specificity of DNA in *Haemophilus influenzae*: The Restriction and Modification Systems in Strains *Rb* and *Rf*

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SUMMARY

Haemophilus influenzae *Rf* possesses two distinct host specificity systems F1 and F2 each of which is able to restrict and modify *Haemophilus* phage HP1c1, while strain *Rb* possesses only one system, B. Among restriction-deficient mutants isolated from strain *Rf*, the $r^{-}m^{+}$ as well as $r^{-}m^{-}$ phenotypes for these two systems were obtained after either one or two mutational steps. The F1 system was introduced into *H. influenzae* *Rd* by genetic transformation to show that the D1 and F1 systems are not allelic.

INTRODUCTION

Many strains of bacteria can recognize DNA from other so-called foreign strains. One result of this recognition is that the foreign DNA molecule can be cleaved by strain specific restriction endonucleases that cut DNA, producing a limited number of fragments (Meselson & Yuan, 1968; Linn & Arber, 1968; Roulland-Dussoix & Boyer, 1969; Smith & Wilcox, 1970). Protection against restriction is provided by another enzyme, modification methylase, that converts a very limited number of adenine or cytosine residues into 6-methylamino-purine and 5-methylcytosine respectively (Smith, Arber & Kühnlein, 1972; Brockes, Brown & Murray, 1972; Hattman, Gold & Plotnik, 1972). In molecular terms a given DNA host-specificity system can be defined by the sequence of base pairs recognized by the restriction endonuclease and modification methylase. The sequence of base-pairs recognized by restriction enzyme isolated from *Haemophilus influenzae* *Rd* and by two R factor restriction enzymes have already been determined (Kelly & Smith, 1970; Hedgpeth, Goodman & Boyer, 1972; Bigger, Murray & Murray, 1973).

The restriction and modification properties of *Haemophilus influenzae* have been characterized *in vitro* (Smith & Wilcox, 1970; Gromkova & Goodgal, 1972; Gromkova, Bendler & Goodgal, 1973; P. Roy, personal communication) as well as *in vivo* (Glover & Piekarowicz, 1972; Piekarowicz & Glover, 1972). Piekarowicz & Glover (1972) have shown that each of the serological types of *H. influenzae* carry different DNA restriction and modification systems. Moreover, strain *Ra* carries two genetically distinct host-specificity systems each of which is able to restrict *Haemophilus* phages HP1c1 and S2.

In this paper we describe the isolation and characterization of restriction-deficient mutants for host specificities in *Haemophilus influenzae* *Rf* and *Rb*. The accompanying paper is concerned with the similarity between *H. influenzae* *Re* and *Rf* host-specificity systems.

Table 1. *Strains of Haemophilus influenzae*

Strain designation	Characteristics	Source	Reference
<i>H. influenzae</i> rd30	Streptomycin resistant	—	Glover & Piekarowicz (1972)
<i>H. influenzae</i> rd123	Streptomycin resistant	—	Glover & Piekarowicz (1972)
<i>H. influenzae</i> rd441	Streptomycin resistant	—	Glover & Piekarowicz (1972)
<i>H. influenzae</i> rf	Wild-type	Dr G. Leidy	—
<i>H. influenzae</i> rf	Novobiocin resistant	Obtained in this laboratory	—
<i>H. influenzae</i> rd441-I	Streptomycin resistant HP1c1 lysogen	Obtained in this laboratory	—
<i>H. influenzae</i> re	Wild-type	Dr G. Leidy	—
<i>H. influenzae</i> rb	Wild-type	Dr G. Leidy	—

METHODS

Bacterial strains. The strains of *Haemophilus influenzae* used are listed in Table 1. Bacterial cultures were routinely stored at -45°C in supplemented BHI medium (see Media) containing 15% (v/v) glycerol. For each experiment samples were thawed, diluted in fresh medium and cultures grown at 37°C on a shaker until they reached titres of 5×10^8 to 1×10^9 bacteria/ml.

Bacteriophages. The phage HP1c1 was originally isolated by Harm & Rupert (1963). Phage S2 was isolated by Bendler & Goodgal (1968). These phages were kindly supplied by R. M. Herriott and J. W. Bendler respectively.

Media. Supplemented BHI was 3.7% (w/v) Brain Heart Infusion (BHI) (Difco) supplemented with $10\text{ }\mu\text{g}$ haemin/ml plus $2\text{ }\mu\text{g}$ NAD/ml. Other media and salt solutions were as described elsewhere (Piekarowicz & Glover, 1972; Spencer & Herriott, 1965).

Phage techniques. The general techniques were as described by Adams (1959) and special techniques relating to HP1c1 phage are those described by Harm & Rupert (1963) and for phage S2 as described by Bendler & Goodgal (1968).

Mutagenesis. Bacteria were mutagenized with *N*-methyl-*N*-nitroso-*N*-nitroguanidine according to the procedure of Piekarowicz & Glover (1972).

Isolation of restriction-deficient mutants. For the isolation of mutants, samples of mutagenized bacteria were thawed and plated at appropriate dilution on supplemented BHI medium and incubated at 37°C . Colonies were then picked into 1 ml supplemented BHI medium and incubated for 16 h at 37°C without aeration. Each culture was then used in a qualitative test for restriction and modification.

Spot test for restriction and modification. These methods were in general as described previously (Piekarowicz & Glover, 1972).

Quantitative test for restriction and modification. Restriction and modification were accurately assayed by determining the efficiency of plating (e.o.p.) of phage stocks on appropriate strains. Routinely the phages were diluted and mixed with 2×10^8 bacteria/ml of the indicator strain in 2.5 ml soft BHI agar containing CaCl_2 (0.001 M) at 43 to 45°C and poured over 10 to 15 ml of supplemented BHI agar also containing 0.001 M- CaCl_2 . Plaques were counted after 16 to 24 h incubation at 37°C .

One-cycle phage infection. These techniques were reported earlier (Piekarowicz & Glover, 1972).

Isolation of DNA. DNA was isolated and purified by the method of Marmur (1961) except that the final step of precipitation with isopropanol was omitted.

Table 2. Efficiency of plating of phages HP1c1 and S2 on strains of *Haemophilus influenzae*

Phage grown on <i>Haemophilus influenzae</i> strains	Rf	Rd30	Rd123
HP1c1.Rd30*	1×10^{-9}	1	1×10^{-8}
HP1c1.Rd123†	1×10^{-9}	1	1
HP1c1.Rf	1	1×10^{-1}	1×10^{-3}
S2.Rd30	1×10^{-9}	1	1×10^{-8}

* Rd30 is an r⁻m⁻ strain of *H. influenzae* rd (Glover & Piekawicz, 1972).

† Rd123 is an r₅m₅⁺ strain of *H. influenzae* rd (Glover & Piekawicz, 1972).

Competence. The preparation of competent *Haemophilus influenzae* rd cultures was based on the method of Spencer & Herriott (1965). The sample of supplemented BHI culture (5×10^8 cells/ml) was centrifuged and washed once with M-II medium and then resuspended in the same volume of M-IV medium. Competence was allowed to develop for 90 min at 37 °C.

Transformation. The general transformation techniques were essentially those of Goodgal & Herriott (1961). A 0.1 ml sample of DNA isolated from a *Haemophilus influenzae* Rf novobiocin-resistant strain of 0.1 ml of competent strain Rd441 (HP1c1) were mixed in 2.8 ml of BHI and incubated for 120 min at 37 °C. The transformed cells were poured directly onto BHI medium containing 2.5 µg novobiocin/ml and incubated 24 h at 37 °C to give colonies of transformants.

Isolation of *Haemophilus influenzae* Rd strain with an Rf host specificity system. To eliminate non-competent cells from the screening procedure (Michalka & Goodgal, 1969), a sample of 0.1 ml DNA (1 µg) from *H. influenzae* Rf resistant to 2.5 µg novobiocin/ml and 0.1 ml DNA (10 µg) from wild-type *H. influenzae* Rf were mixed with 0.1 ml of competent strain Rd441 (HP1c1) in 2.8 ml BHI and incubated for 120 min. The transformants were poured directly into 2.5 ml soft agar on the surface of 10 ml hard agar containing 2.5 µg novobiocin/ml and the plates were incubated overnight at 37 °C to give small colonies. To detect clones transformed to donor type (Rf) host specificity the colonies were overlayed with the following indicator strains: *H. influenzae* Rf wild-type, Rf232 and Rf101 in soft agar. Colonies spontaneously releasing HP1c1 phage with modification specific for *H. influenzae* Rf formed haloes of lysed cells in the top layer containing the appropriate indicator bacteria. They were stabbed and streaked on antibiotic-containing agar to eliminate sensitive indicator bacteria. Single colonies were grown for further examination and frozen in broth containing 15 % (v/v) glycerol.

RESULTS

Analysis of restriction and modification properties of Haemophilus influenzae Rf

Piekawicz & Glover (1972) showed that unmodified phage HP1c1.O is restricted by *Haemophilus influenzae* Rf with a very high efficiency (e.o.p. about 10^{-9}). To ensure that the observed low e.o.p. of phage HP1c1 on strain Rf was due to restriction and modification, two experiments were carried out. In the first experiment we checked the low efficiency of plating of phage HP1c1.O by measuring restriction in supplemented BHI cultures. A sample of 5×10^9 *H. influenzae* Rf/ml was infected with HP1c1.O phage at a multiplicity of infection of 2.0 and after 15 min for adsorption a large number of samples were plated on *H. influenzae* Rf as an indicator strain. This procedure almost completely eliminated plaques arising from unadsorbed phage but still allowed the permissive fraction

Table 3. *One-cycle infection of Haemophilus influenzae Rd with phage HP1c1.Rf*

No. bacteria/ml	8×10^8
No. phages/ml	1.3×10^9
Multiplicity of infection	0.16
Adsorption	99 %
No. infective centres on:	
Rd30	2.5×10^8
Rf	1.2×10^8
Transmission coefficient	2×10^{-1}
Progeny phage on:	
Rd30	1.3×10^{10}
Rf	1.0×10^7
Average burst size	52
Percentage of progeny phage which plate on Rf	0.1

of infected bacteria to yield plaques. The results in Table 2 confirmed our previous observations on the very high efficiency of restriction of HP1c1 and S2 phages in *H. influenzae* strain Rf.

In the second experiment, one-cycle phage infection was carried out using phage HP1c1.Rf to infect an r⁻m⁻ strain of *H. influenzae* Rd. Phage HP1c1.Rf can infect the r⁻m⁻ strain efficiently and a major part of the progeny phage is unable to form plaques on the restricting indicator strain Rf, indicating that the ability to form plaques on Rf is not heritable (Table 3).

Isolation of restriction-deficient mutants of Haemophilus influenzae Rf

Piekarowicz & Glover (1972) showed that the low e.o.p. of phage HP1c1.O on *Haemophilus influenzae* Ra (about 10^{-6}) is due to the activity of two systems, A1 and A2, which reduce the e.o.p. to 3×10^{-9} and 5×10^{-3} respectively. *Haemophilus influenzae* Rd possesses only one detectable host specificity system (Glover & Piekarowicz, 1972) which reduces the e.o.p. to 1×10^{-3} . In the light of these results the very low e.o.p. of HP1c1.O on Rf strain makes it likely that this strain has two or more host-specificity systems.

The restriction-deficient mutants of *Haemophilus influenzae* Rf were isolated following NTG mutagenesis as described in Methods. In Expt I (Table 4) a total of 8 mutants was isolated from about 250 colonies and in Expt II a total of 14 mutants was isolated from about 300 colonies. None of the restriction-deficient mutants isolated is completely permissive for phage HP1c1.O. The great majority of them (20 out of 22) are able to host-modify phage HP1c1 so that it can plaque on strain Rf with an efficiency of 1.0, but the other two cannot. None of them is fully m⁻, since phage from these two mutants plaques at higher efficiency on Rf than does HP1c1.O. The mutants have thus either an r[±]m⁺ or an r[±]m[±] phenotype. One obvious explanation of these results would be that strain Rf possesses more than one genetically independent system of restriction and modification.

Two restriction and modification systems in Haemophilus influenzae Rf

To test the possibility that *Haemophilus influenzae* Rf, like strain Ra, possesses more than one restriction and modification system, phage HP1c1 was grown on each of the mutants and its e.o.p. on each of the other mutants was measured (Table 5). The simplest explanation

Table 4. *Restriction and modification properties of restriction-deficient mutants of Haemophilus influenzae Rf*

Expt no.	Mutant no.	Restriction e.o.p. of HP1c1.O* on mutant	Modification e.o.p. of progeny phage HP1c1.mutant on Rf†
I	101	2×10^{-4}	2×10^{-6}
	102	4×10^{-4}	I
	103	5×10^{-7}	I
	104	1×10^{-6}	I
	106	1×10^{-7}	I
	109	1×10^{-7}	I
	111	5×10^{-7}	I
	113	2×10^{-7}	I
II	214	1×10^{-7}	I
	215	1×10^{-7}	I
	217	1×10^{-6}	I
	218	1×10^{-7}	I
	220	1×10^{-3}	I
	221	1×10^{-7}	I
	222	2×10^{-6}	I
	225	1×10^{-7}	I
	227	1×10^{-3}	I
	229	1×10^{-5}	I
	230	1×10^{-7}	I
	231	1×10^{-7}	I
	232	1×10^{-6}	2×10^{-3}

The mutants were isolated after NTG treatment as described in Methods.

Expt I: NTG 300 µg/ml, treatment for 30 min at 37 °C, 0.1 % survivors.

Expt II: NTG 300 µg/ml, treatment for 30 min at 37 °C, 0.06 % survivors.

* HP1c1.O was phage grown on *H. influenzae* rd30 (Glover & Piekawicz, 1972).

† The e.o.p. of progeny phage HP1c1 mutant on all mutants was 1.

Table 5. *Efficiency of plating on restriction-deficient mutants of Haemophilus influenzae Rf of phage HP1c1 previously grown on the mutants*

Host strain	Previous host for phage HP1c1				Host specificity phenotype
	HP1c1.Rf101	HP1c1.Rf102	HP1c1.Rf232	HP1c1.Rf217	
Rf101	I	I	1×10^{-4}	I	$r_{F1}^+ m_{F1}^- r_{F2}^+ m_{F2}^+$
Rf102	I	I	1×10^{-4}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf103	1×10^{-7}	I	3×10^{-4}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf104	3×10^{-5}	I	3×10^{-3}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf106	3×10^{-5}	I	3×10^{-3}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf111	3×10^{-5}	I	3×10^{-3}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf217	1×10^{-6}	I	I	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf220	I	I	3×10^{-3}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf222	I	I	3×10^{-3}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf227	I	I	3×10^{-3}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf229	1×10^{-7}	I	2×10^{-3}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
Rf232	1×10^{-6}	I	I	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$
RfW.t.	1×10^{-6}	I	3×10^{-3}	I	$r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$

Table 6. *Double restriction-deficient mutants isolated from Haemophilus influenzae* rf232

Cultures of *H. influenzae* rf232 were treated with a final concentration of 300 µg NTG/ml for 30 min at 37 °C to give a survival of 0.01 %, as described in Methods. A total of 350 colonies was screened for restriction-deficient mutants with phage HP1c1.O.

Previous host for phage HP1c1	Efficiency of plating on <i>Haemophilus influenzae</i> rf mutants			
	rf232-105	Rf	rf232	Rf101
HP1c1.Rf232-105	1	1×10^{-9}	1×10^{-6}	5×10^{-3}
HP1c1.O*	1	1×10^{-9}	1×10^{-6}	1×10^{-4}

* Phage HP1c1 previously grown on strain rd30 (Glover & Piekarowicz, 1972).

Table 7. *Double restriction-deficient mutants isolated from Haemophilus influenzae* rf101

A culture of *Haemophilus influenzae* rf101 was treated with a final concentration of 300 µg NTG/ml for 30 min at 37 °C to give a survival of 0.01 %, as described in Methods. A total of 250 colonies was screened for restriction-deficient mutants with phage HP1c1.O.

Mutant no.	Restriction e.o.p. of HP1c1.O*	Modification e.o.p. of progeny phage HP1c1.mutant on Rf101†	Phenotypes of mutants deduced
	on mutants		
Rf101-8	1×10^{-1}	1	$r^{\pm}m^{+}$
Rf101-22	1	4×10^{-2}	$r^{-}m^{\pm}$
Rf101-26	1×10^{-1}	1	$r^{\pm}m^{+}$
Rf101-82	1	1×10^{-4}	$r^{-}m^{-}$
Rf101-117	1	1	$r^{-}m^{+}$

* Phage previously grown on strain rd30 (Glover & Piekarowicz, 1972).

† The e.o.p. on all mutants was 1.

of these data is that mutants rf232 and Rf101 possess independent restriction and modification systems each one representing one of the two systems which are present in the wild-type *H. influenzae* rf. We shall denote these systems F1 and F2 so that the phenotype of rf232 can be written $r_{F1}^{+}m_{F1}^{+}r_{F2}^{-}m_{F2}^{-}$ and the phenotype of Rf101 can be written $r_{F1}^{-}m_{F1}^{-}r_{F2}^{+}m_{F2}^{+}$. The phenotypes of the other mutants can be deduced from whether they restrict phage grown on rf232 (i.e. HP1c1.F1, O) in which case they are r_{F2}^{+} or whether they restrict HP1c1.O, F2 (i.e. phage grown on Rf101) in which case they are r_{F1}^{+} . It is clear from the results presented in Table 5 that for both systems many of the mutants expressed phenotypes intermediate between + and - for restriction and also for modification. Most of them are characterized by changes in the F1 system, perhaps because the screening procedure favours the isolation of this class of mutants. The e.o.p. of phage HP1c1.O on r_{F1}^{+} strains is about 1×10^{-6} while the e.o.p. of phage HP1c1.O on r_{F2}^{+} strains is much higher, about 2×10^{-3} .

Double mutants isolated for restriction-deficiency from Haemophilus influenzae Rf

The hypothesis that *Haemophilus influenzae* Rf possesses two genetically distinct systems of restriction and modification was tested following the isolation of double restriction-deficient mutants from rf232 and Rf101 strains. If this hypothesis is correct, then it should be possible to isolate mutants with different phenotypes for these two systems and certain classes of mutants would be expected to occur only as a result of two mutations.

The Rf232 and Rf101 strains were treated with NTG and the survivors cloned and screened

Table 8. One-cycle phage infection on *Haemophilus influenzae* Rf101

Growth of phage HP1c1.Rf232 in strain Rf101.

No. bacteria/ml	8×10^8
No. phages/ml	7×10^6
Multiplicity of infection	0.01
Adsorption	99 %
No. infective centres on:	
Rf101	1.5×10^4
Rf232	1.0×10^4
Rf	6.0×10^3
Transmission coefficient	2×10^{-3}
Progeny phage plated on:	
Rf101	1.4×10^6
Rf232	1.4×10^4
Rf	1.2×10^4
Average burst size	95
Percentage of progeny phage which plate on:	
Rf101	100
Rf232	1
Rf	0.9

for restriction-deficient mutants. Several double mutants were obtained and their restriction and modification properties are summarized in Tables 6 and 7. Only one mutant which was completely restriction-deficient was obtained from strain Rf232. This mutant accepts phage HP1c1.O at an e.o.p. of 1.0 and the phage propagated on this strain totally lacks Rf modification. The phenotype of this double mutant is therefore $r_{F1}^-m_{F1}^-r_{F2}^-m_{F2}^-$. Three completely restriction-deficient double mutants were obtained after mutagenesis of strain Rf101. However, they differ in modification. One of them, Rf101-82, is completely modification deficient, another, Rf101-117, has the same modification properties as the parental strain Rf101, while the third, Rf101-22, is intermediate. The phenotypes of these double mutants are therefore $r_{F1}^-m_{F1}^-r_{F2}^-m_{F2}^-$ (Rf101-82), $r_{F1}^-m_{F1}^-r_{F2}^+m_{F2}^+$ (Rf101-117) and $r_{F1}^-m_{F1}^-r_{F2}^+m_{F2}^+$ (Rf101-22). Mutants with the phenotype $r_{F2}^+m_{F2}^+$ were also isolated (see Table 7).

The properties of double mutants isolated for their restriction-deficiency from one-step mutants Rf232 and Rf101 confirm the hypothesis that *Haemophilus influenzae* Rf possesses two separate host-specificity systems. For each host specificity system the phenotypes r^-m^+ and r^-m^- were obtained in approximately equal numbers. All of the Rf one-step mutants which were obtained were mutated in one or other host specificity but not in both. Strains defective in both systems were obtained only as a result of two mutational events. Like the host-specificity systems in strain Ra (Piekarowicz & Glover, 1972) and unlike the host-specificity system in strain Rd (Glover & Piekarowicz, 1972) the two host-specificity systems F1 and F2 are stable and the mutants do not revert at high frequency.

One cycle phage infection experiments with Rf host specificity systems F1 and F2

The restriction and modification properties of two host-specificity systems F1 and F2 in *Haemophilus influenzae* Rf were examined in a series of one-cycle phage infection experiments. There was no difference in adsorption or burst size for fully modified phage HP1c1.Rf

Table 9. *Efficiency of plating of phage HP1c1.Rd441-TF1 on strains of Haemophilus influenzae*

Strain	E.o.p. of HP1c1.Rd441-TF1
<i>rf</i> wild-type	1×10^{-3}
<i>rf</i> 232 ($r_{F1}^+ m_{F1}^+ r_{F2}^- m_{F2}^-$)	1
<i>rf</i> 101 ($r_{F1}^- m_{F1}^- r_{F2}^+ m_{F2}^+$)	1×10^{-3}
<i>rd</i> 123 ($r_{D1}^+ m_{D1}^+$)	1
<i>rd</i> 441 ($r_{D1}^- m_{D1}^+$)	1

between the wild-type strain *rf* and the mutants *rf*232 and *rf*101 which possess only the F1 and F2 systems respectively. The transmission coefficient (i.e. the fraction of infected bacteria which produce progeny phage) for HP1c1.O in *rf*232 r_{F1}^+ is approximately 10^{-6} and the transmission coefficient for phage HP1c1.O in *rf*101 r_{F2}^+ is approximately 10^{-3} . Together, these account entirely for the efficiency of plating of HP1c1.O on strain *rf* r_{F1}^+ , r_{F2}^+ .

Interesting confirmation of previous results was obtained in the one-cycle infection experiments with phage HP1c1.F1 in strain *rf*101 $r_{F1}^- m_{F1}^- r_{F2}^+ m_{F2}^+$. If *Haemophilus influenzae rf* wild-type like strain *Ra* possess two independent host-specificity systems, whereas mutant *rf*101 has only one, then phage HP1c1 carrying one type of modification grown in a strain possessing the second host-specificity system should yield among the progeny a few phage particles carrying both modifications HP1c1.F1,F2 (Kellenberger, Symonds & Arber, 1966; Piekarowicz & Glover, 1972). These particles originating from infection with HP1c1.F1 should, and did, plaque equally well on strain *rf* and *rf*101 (Table 8).

Transfer of the host specificity systems of Haemophilus influenzae rf to H. influenzae strain rd

If the analysis of the host-specificity phenotypes of restriction-deficient mutants of *rf* demonstrating the existence of two independent host-specificity types in this strain is correct, then it should be possible to separate them genetically by transformation. Since *Haemophilus influenzae rd* is the most readily transformable strain, the *rd*441 mutant $r_{D1}^- m_{D1}^+$ (Glover & Piekarowicz, 1972) lysogenic for HP1c1 phage was used as a recipient in these experiments. Integration of *H. influenzae rf* DNA into the genome of *H. influenzae rd* is as efficient as for *H. influenzae rd* DNA (unpublished observations). Strain *rd*441 was transformed with a mixture of *rf nov-r* and *rf* wild-type DNA. Novobiocin-resistant transformants were isolated and screened for *rf* host specificity. This selection procedure was based on the assumption that cells possessing *rf* host specificity should release phage able to plaque on either *rf*232 or *rf*101 indicator strains. However, in practice the transformed cells released so many phage particles that *rf*101 F1⁺ was not an effective indicator strain.

Among several thousands of novobiocin-resistant transformants of *rd*441 (HP1c1) one was isolated with released phage HP1c1 which plaqued equally well on *rd*441 and *rf*232 indicator bacteria. The properties of phage HP1c1 released by this particular transformant *rd*441-TF1 (shown in Table 9) indicate that *rd*441-TF1 possesses two host-specificity systems, one specific for *rd* and the other specific for one of the systems present in *Haemophilus influenzae rf*, the F1 system. This experiment thus confirms the results of genetical analysis of restriction-deficient mutants of *H. influenzae rf* and demonstrates the existence of two independent host-specificity types in this strain. In addition it can be argued that the *hs* loci

Table 10. Restriction-deficient mutants of *Haemophilus influenzae* Rb.

Expt no.	Mutant no.	Restriction e.o.p. of HP1c1.O* on mutant	Modification e.o.p. of progeny phage HP1c1 mutant on:	
			Rb	Mutant
I	188	1.8×10^{-1}	1.0	1.0
	116	5.0×10^{-3}	1.0	1.0
II	218	5.8×10^{-1}	1.0	1.0
	220	1.0	1.0	1.0
	234	5.0×10^{-1}	1.0	1.0
III	None	—	—	—
IV	435	1.3×10^{-1}	1.0	1.0
	437	1.0	3.4×10^{-4}	1.0

The mutants were isolated after NTG treatment as described in Methods.

Expts I and II: NTG 300 µg/ml, treatment for 15 min at 37 °C, 0.01 % survival.

Expts III and IV: NTG 400 µg/ml, treatment for 20 min at 37 °C, 0.001 % survival.

* HP1c1.O was phage grown on *H. influenzae* Rd30 (Glover & Piekawicz, 1972).

Table 11. Efficiency of plating on restriction-deficient mutants of *Haemophilus influenzae* Rb of phage HP1c1 previously grown on the mutants

Previous host for phage HP1c1	Strain								
	Rd30	Rb	Rb188	Rb116	Rb218	Rb220	Rb234	Rb435	Rb437
Rd30	1.0	1.7×10^{-4}	1.8×10^{-1}	5×10^{-3}	5.8×10^{-1}	1.0	5×10^{-1}	1.3×10^{-1}	1.0
Rb	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rb188	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rb116	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rb218	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rb220	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rb234	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rb435	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rb437	1.0	3.4×10^{-4}	6.2×10^{-2}	6.5×10^{-3}	1.0	1.0	1.0	8×10^{-2}	1.0
Phenotype deduced		r+m+	r±m+	r±m+	r-m+	r-m+	r-m+	r±m+	r-m-

of the D and F1 systems are not allelic since they are both present in the transformant Rd441-TF1.

Isolation of restriction-deficient mutants of *Haemophilus influenzae* Rb

Piekawicz & Glover (1972) showed that unmodified phage HP1c1.O is restricted by *Haemophilus influenzae* Rb with e.o.p. about 10^{-4} . In the light of the results of genetical analysis of host specificity in *H. influenzae* Rd (Glover & Piekawicz, 1972), Ra (Piekawicz & Glover, 1972) and in Rf as shown above, an e.o.p. of 10^{-4} for HP1c1.O on strain Rb indicates that this strain may have only one host-specificity system. To test this possibility restriction-deficient mutants of *H. influenzae* Rb were isolated and their phenotypes tested.

The restriction-deficient mutants of *Haemophilus influenzae* Rb were isolated following NTG mutagenesis (Table 10). Among several restriction-deficient mutants only two were completely permissive for HP1c1.O. However, they differ in modification. One of them, Rb437, is completely modification-deficient and the other, Rb220, has the same modification

properties as the parental strain *Rb*. The phenotypes of these mutants are therefore $r_B^- m_B^-$ and $r_B^- m_B^+$. Mutants with the phenotype $r_B^+ m_B^+$ were also isolated.

The results of the chequer-board experiment presented in Table II demonstrate that *Haemophilus influenzae* *Rb* possesses only one restriction and modification system since none of the mutants compared pair-wise show mutually exclusive restriction and modification properties.

DISCUSSION

Strain *Haemophilus influenzae* *Rf* carries two DNA host-specificity systems which were designated F1 and F2. The host specificity phenotype of *H. influenzae* *Rf* is therefore $r_{F1}^+ m_{F1}^+ r_{F2}^+ m_{F2}^+$. Results of the following experiments argue for their independence. Analysis of the restriction-deficient mutants of *Rf* revealed strains possessing only one host-specificity type. Most of the predictable phenotypes for mutants of these two systems were obtained after mutagenesis. All of the mutants isolated after a single mutagenic treatment was affected in a single host-specificity system, i.e. either F1 or F2 but never in both. Double mutants affected in both systems were only obtained after mutagenic treatment of previously isolated mutants defective in either F1 or F2. The two host-specificity systems of *Rf* were separated by transformation. Using donor DNA from *H. influenzae* *Rf* and *H. influenzae* *Rd* as a recipient, a transformant possessing the recipient D and the donor F1 systems was obtained. The donor F2 system was not inherited by this transformant.

The efficiency of plating of phage HP1C1.O on *Haemophilus influenzae* *Rf* is reduced to an extremely low level, 1×10^{-9} , as a result of the activity of these two systems, which are roughly additive. The F1 system reduces the e.o.p. to 1×10^{-6} and F2 reduces the e.o.p. to 1×10^{-3} . The level of restriction expressed by the F1 system is one of the highest for a single host-specificity system known so far. Within limits, the restriction coefficient for a given phage by a given system is a measure of the number of sites present on the phage DNA (Franklin & Dove, 1969; Murray, Manduca de Ritis & Foster, 1973). Phage *f1*.O for example, which has two sites for the B system, has an e.o.p. 10^{-4} on *Escherichia coli* B (Boon & Zinder, 1971) but mutants which have lost either of two *sB* sites are restricted with an e.o.p. 10^{-2} (Lyons & Zinder, 1972). Thus, the e.o.p. of 10^{-6} observed for phage HP1C1 in the F1 system may reflect the presence of several sensitive sites on its DNA. On the other hand, this high level of restriction may mean that F1 restriction endonuclease is present in larger amounts than other endonucleases, or it is more efficient, or it is not subject to the physiological influences which frequently affect the fraction of permissive cells in a population.

Genetical analysis of the host-specificity types in *Haemophilus influenzae* strains has revealed that the e.o.p. of unmodified HP1, HP1C1 and S2 phages is related to the number of host-specificity types carried by these strains. Thus, the e.o.p. of phage HP1C1.O on *H. influenzae* *Ra* is reduced to 5×10^{-6} as a result of the activity of two systems A1 and A2 (Piekarowicz & Glover, 1972), the e.o.p. of phage HP1C1.O on *H. influenzae* *Rd* is 10^{-3} (Glover & Piekarowicz, 1972) and on *Rb* is about 10^{-4} , and both these strains have only one system of host specificity. The extremely low e.o.p. on *Rf* and *Re* strains is due to two independent systems present in these strains.

We do not know whether these two systems are determined by chromosomal or plasmid genes.

The phenotypes of restriction-deficient mutants F1 and F2 fall into two classes in respect of modification; those which are modification-deficient and those which are modification-proficient. These results are compatible with the three-gene model of restriction and modification (Arber & Linn, 1969; Glover, 1970; Boyer, 1971). According to this model, $r^- m^+$

mutants arise as a result of mutations in a gene *hsr* which is necessary for restriction, and $r^{-}m^{-}$ mutants are due to mutations in a gene *hss* which is necessary for restriction and modification. Mutations in *hsm* also affect restriction and modification (Hubacek & Glover, 1970). The fact that we isolated $r^{-}m^{-}$ and $r^{-}m^{+}$ one-step mutants for both the F1 and F2 systems could be taken to mean that these host specificities are each controlled by a set of three genes. Thus, it would appear that all the known systems of restriction and modifications in *Haemophilus influenzae* characterized genetically can be classified as type I according to the classification of host-specificity systems by Boyer (1971). However, it cannot be ruled out that in these *Haemophilus* systems only two genes are involved, one of them essential for modification but both of them essential for restriction. The recent isolation of an endonuclease from Rd by Gromkova *et al.* (1973) which is S-adenosyl-methionine-dependent would indicate that the r_D^{+} system is type I.

It is well established that the genes controlling K, B and A restriction and modification in *Escherichia coli* are allelic (Boyer, 1964; Colson, Glover, Symonds & Stacey, 1965; Lederberg, 1966; Wood, 1966; Arber & Wauters-Willems, 1970). The close relatedness of these genes is also reflected in the functional complementation found for some of their products (Glover, 1968; Boyer & Roulland-Dussoix, 1969; Arber & Linn, 1969). No such analysis can be carried out for *Haemophilus influenzae* host specificities; nevertheless it seems that at least some of them are not allelic. The fact that we were able to isolate a strain carrying both the D and F1 systems shows that these two systems are not allelic. These two systems differ also in expression. It is known that the D system is very unstable (Glover & Piekarowicz, 1972) in contrast to any other *H. influenzae* host-specificity system (Piekarowicz & Glover, 1972; Piekarowicz & Kalinowska, 1973). Moreover, in the strain carrying both the D and F1 systems D is still unstable while F1 is stable (unpublished observations).

The fact that $r^{-}m^{+}$ and $r^{-}m^{-}$ mutants were obtained for each host-specificity system in strains which were wild type for the second host-specificity system makes it unlikely that the two systems of *Haemophilus influenzae* r_f are functionally related and cannot like *Escherichia coli* K and B types complement one another by exchange of subunits. The genetic analysis of *H. influenzae* has inherent limitations which do not allow complementation tests to be carried out, so that it is not possible to establish directly the relationship between the different host-specificity systems present in these bacteria.

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