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Incompatibility and Surface Exclusion Properties of H_1 and H_2 Plasmids

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Plasmids of the H incompatibility group showed two types of surface exclusion and incompatibility interactions. Strong incompatibility and surface exclusion were evident between plasmids within the same subgroup, and recombination frequently occurred between these plasmids after antibiotic selection for the presence of two plasmids in the same cell. Weaker interactions were seen between plasmids of the different subgroups, H_1 and H_2, and recombination was not detected. Incompatibility between H_1 and H_2 plasmids led preferentially to the loss of the H_1 plasmid, irrespective of the order of entry of the plasmids. These data are consistent with the hypothesis that incompatibility is negatively controlled.

Surface exclusion and incompatibility are two important properties that are often used to determine whether plasmids are related (5). However, only a few investigators attempted to analyze incompatibility qualitatively (6, 22). Plasmids belonging to the H incompatibility group (12) present a unique situation for such analysis. Two subgroups, H_1 and H_2, are recognized. Members of both subgroups exhibit thermosensitive mating and are incompatible (1, 20); however, representative plasmids from each group have little or no deoxyribonucleic acid homology with each other (13). Division into H_1 and H_2 depends on the incompatibility of the H_1 plasmid with an autonomous F factor (19); H_2 plasmids are compatible with F. Members of the H_2 subgroup have also been shown to inhibit the development of several double-stranded deoxyribonucleic acid phages, such as λ, T1, T5, and T7 (21).

Two types of incompatibility interactions may be studied by using H plasmids: "homo-incompatibility" between members of the same subgroup, and "hetero-incompatibility" between members of different subgroups. At present, the H group of plasmids is the only one that consists of two clearly distinguishable subgroups in which members are incompatible. In this communication, we report the quantitative measurement of surface exclusion and incompatibility both within and between these subgroups.

MATERIALS AND METHODS

Bacterial strains and plasmids. The two bacterial strains, RG176 (nalidixic acid resistant) and RG488 (rifampin resistant, lactose nonfermenting), used for surface exclusion and incompatibility testing were derived from a prototrophic, lactose-fermenting *Escherichia coli* C strain (11). The H plasmids used in this study are shown in Table 1. Plasmids that do not confer resistance to tetracycline or chloramphenicol were prepared by mutagenesis with ethyl methane sulfonate.

Measurement of surface exclusion. The frequencies of transfer of the H plasmids from related strains of *E. coli* (RG176 and RG488) to R^- and R^+ strains of the reciprocal host were determined at 26°C after a 16-h mating period by the method of conjugation described previously (11). The mating mixtures were plated on MacConkey agar (Difco) that contained antibiotics—either rifampin (25 μg/ml) or nalidixic acid (24 μg/ml), and either chloramphenicol (16 μg/ml) or tetracycline (8 μg/ml). Selection was for the chromosomal marker and for a resistance of the incoming plasmid only. The relative frequency of transfer by conjugation to strains of *E. coli* C with and without a second H plasmid gave a measure of the surface exclusion (17).

Measurement of incompatibility. A modification of the colony test (2, 22) was used to give a quantitative estimate of the degree of incompatibility. Two methods were used to prepare transconjugant clones: antibiotic selection for the entering plasmid only, and double-antibiotic selection for both plasmids with chloramphenicol (16 μg/ml) and tetracycline (8 μg/ml). Colonies were picked, diluted in 0.05 M sodium phosphate buffer, pH 7.2, and plated on nonselective medium (MacConkey agar). After 16 h of incubation at 37°C, 100 colonies were picked with toothpicks to nonselective agar and, after another 16 h of incubation at 37°C, the colonies were replica plated onto agar containing tetracycline or chloramphenicol. After an additional 8 h of incubation at 37°C, the number of colonies containing one or both plasmids was recorded.

TABLE 1. *H plasmids employed in this study*

Plasmid designation	Incompatibility subgroup	Resistance pattern[a]	Source or reference
pRG1251[b]	H_1	Ap Cm Sm Sp Su Tc	Taylor and Grant (submitted for publication)
pRG1251-1	H_1	Ap Cm Sm Sp Su	pRG1251
pRG1251-2	H_1	Ap Sm Sp Su Tc	pRG1251
pAS251-2	H_2	Cm Km Sm Tc	11
pAS251-2-1[c]	H_2	Cm Km Sm	21
pSD 114	H_2	Cm Km Sm Tc	21

[a] Ap, Ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamide; Tc, tetracycline.

[b] Originally isolated from *Salmonella typhi* from Thailand (20).

[c] Previously designated pAS251-2T$^-$ (21).

The kinetics of incompatibility between pRG1251-2 (H_1) and pAS251-2-1 (H_2) were measured with cells from clones isolated by double-antibiotic selection that were suspended in 1 ml of Penassay broth (Difco antibiotic medium no. 3) and allowed to grow at 37°C. The cell density was kept below 10^7 cells per ml by diluting the cultures periodically in prewarmed Penassay broth. Samples were withdrawn throughout the incubation period and plated on nonselective agar. The nature of the plasmids remaining at each time was determined by toothpicking 100 colonies to MacConkey agar and, subsequently, replica plating on chloramphenicol (to identify pAS251-2-1) or ampicillin and tetracycline (to identify pRG1251-2).

RESULTS

A surface exclusion hierarchy was evident between H plasmids pairs, with H_1-H_1 exclusion (pRG1251-2 and pRG1251-1) being stronger than that between H_2 plasmids, whereas interactions between H_1 and H_2 were the weakest (Table 2). There was little difference in exclusion between pairs of H_2 plasmids that are "cogenic" (pAS251-2 and pAS251-2-1) and between different isolates from the same geographic locality (pSD114 and pAS251-2-1).

Results of the colony tests for incompatibility between H_1 plasmids are shown in Table 3. When doubles were prepared by selecting for the incoming plasmid marker and for the chromosomal marker, in most cases the resident plasmid was displaced by the newly selected plasmid. Only one of 20 clones so prepared contained both plasmids (Table 3, experiment 1), and the plasmids in this clone segregated after further growth of the culture on nonselective medium.

To try to measure the segregation rate of the H_1 plasmids in incompatibility tests between two H_1 plasmids, as Uhlin and Nordström (22) have done for FII plasmids, we selected for both plasmids simultaneously by using chloramphenicol to select for one plasmid and tetracycline to select for the other. Twenty clones were tested. Each contained both resistance markers, and the markers were stably inherited during subsequent growth in nonselective medium. Mating experiments showed that both markers had become genetically linked. We conclude, therefore, that forcing the two H_1 plasmids to coexist resulted in the formation of recombinant plasmids.

TABLE 2. *Surface exclusion between plasmids of the H incompatibility group*

Incoming plasmid	Resident plasmid	Marker selected	Transfer frequency[a]	Surface exclusion index[b]
pRG1251-2 (H_1)	pRG1251-1, (H_1)	Tc[c]	1×10^{-6}	400
pRG1251-2 (H_1)	pAS251-2-1 (H_2)	Tc	1×10^{-4}	10
pAS251-2-1 (H_2)	pRG1251-2 (H_1)	Cm	1×10^{-4}	10
pAS251-2 (H_2)	pAS251-2-1 (H_2)	Tc	1×10^{-4}	100
pSD114, (H_2)	pAS251-2-1 (H_2)	Tc	3×10^{-5}	100

[a] Determined from 16-h mating at 26°C in Penassay broth, measured as number transconjugants per recipient.

[b] Defined as the frequency of transfer of a plasmid to a particular bacterial strain divided by the frequency of transfer of the plasmid under similar conditions to an R$^+$ derivative of the same recipient strain. In the experiments reported here, the donor strain was always an R$^+$ derivative of RG176, and the recipients were RG488 and the appropriate R$^+$ derivatives of RG488.

[c] Abbreviations as in Table 1, footnote *a*.

Incompatibility between H_1 and H_2 plasmids was much weaker than that between the H_1 and H_1 derivatives, and many transconjugant clones contained both plasmids, whether selection was for one or both plasmids (Table 4). The resident plasmid was found to be relatively stable and was not immediately lost in response to antibiotic selection for the incoming plasmid. This stability was especially evident when the resident was H_2 (Table 4, experiment 1). The H_2

TABLE 3. *Colony test for incompatibility between closely related H_1 plasmids*[a]

Expt no.	Incoming plasmid	Resident plasmid	Marker(s) selected	% Daughter colonies containing:[b]		
				Incoming plasmid only	Resident plasmid only	Both plasmids
1	pRG1251-1	pRG1251-2	Cm[c]	100	0	0
2	pRG1251-2	pRG1251-1	Tc	100	0	0
3	pRG1251-1	pRG1251-2	Cm, Tc	0	0	100[d]
4	pRG1251-2	pRG1251-1	Cm, Tc	0	0	100[d]

[a] Transconjugant clones were picked, diluted in 0.05 M sodium phosphate, and grown under nonselective conditions as described in the text. From each clone, 100 daughter colonies were picked and tested for the loss of chloramphenicol resistance (pRG1251-1) and tetracycline resistance (pRG1251-2).

[b] Each experiment was repeated 10 times, and identical results were obtained, except for experiment no. 1. In this experiment, nine clones gave results as shown, but one clone gave rise to daughter colonies, 50% of which contained both plasmids, at the time of the colony test. The two plasmids segregated after further growth on nonselective medium.

[c] Abbreviations as in Table 1, footnote *a*.

[d] Conjugation experiments with representative colonies showed that the plasmids had formed stable recombinants.

TABLE 4. *Colony test for incompatibility between plasmids of the H_1 and H_2 incompatibility groups after selection for the incoming plasmids*[a]

Expt no.	Plasmid		Marker selected	Transconjugant clone no.	% Daughter colonies containing:		
	Incoming[b]	Resident[c]			Incoming plasmid only	Resident plasmid only	Both plasmids
1	pRG1251-2 (H_1)	pAS251-2-1 (H_2)	Tc[d]	1	24	50	26
				2	20	48	32
				3	30	32	38
				4	26	32	42
				5	10	46	44
Avg					22	42	36
2	pAS251-2-1 (H_2)	pRG1251-2 (H_1)	Cm	1	92	0	8
				2	84	0	16
				3	68	8	24
				4	62	0	38
				5	44	4	52
Avg					70	2	28

[a] In each experiment, five transconjugant clones were picked and tested as described in footnote *a* of Table 3. Plasmids pRG1251-2 and pAS251-2-1 were monitored by tetracycline and chloramphenicol, respectively.

[b] Host was RG488.

[c] Host was RG176.

[d] Abbreviations as in Table 1, footnote *a*.

plasmid was invariably the stronger of the pair and was able to remain in the cells, whereas the incoming H_1 plasmid was often lost under the conditions of the colony test. When H_1 was the resident, H_2 was often able to displace H_1 (Table 4, experiment 2). Selection for both plasmids produced a larger number of doubles than selection for the incoming plasmid only.

To determine whether H_1 and H_2 plasmids remained as separate entities after selection for two resistances or whether they recombined, we prepared two clones by selection for both pRG1251-2 and pAS251-2-1. The results of the colony tests with the two clones are shown in Table 5. Cells from both clones were grown separately in Penassay broth for 6 h as described above and tested for the presence of both plasmids with ampicillin and tetracycline resistance (pRG1251-2) and chloramphenicol resistance (pAS251-2-1) (Fig. 1). The clone that contained the H_2 plasmid as the initial resident (Table 5, clone 1) gradually lost the H_1 plasmid over the 6-h period of growth in broth (Fig. 1A). Some of the cells in the clone that contained the H_1 plasmid as resident (Table 5, clone 2) lost the H_1 plasmid, but 44% of the cells

contained both plasmids after 6 h of growth in broth (Fig. 1B). To determine if recombination occurred in clone 2, matings were performed between cells from the broth culture and an R^- derivative of RG488 at 0, 2, 4, and 6 h. Both plasmids were always transferred separately, showing that recombination had not taken place.

DISCUSSION

Our study of incompatibility between R plasmids shows that both strong and weak incompatibility interactions can be found within the H incompatibility group. The homo-incompatibility reactions, those within the H subgroups, are strong. In interactions between two H_1 plasmids, the resident plasmid is rapidly replaced after antibiotic selection for the entering plasmid, but when selection is for both plasmids, recombination occurs. This is also true of incompatibility between H_2 plasmids, as shown by interactions between the plasmid N-1 and another H_2 plasmid (D. E. Taylor and R. B. Grant, Mol. Gen. Genet., in press). In contrast, hetero-incompatibility reactions between the H_1 and H_2 subgroups are weak. Many doubles are formed after antibiotic selection for the entry of a second plasmid or after selection for both plasmids (Table 4). The H_1 and H_2 plasmids do not recombine, and both plasmids can be recovered from single clones. As reported previously (12, 19), H_2 is the dominant plasmid, and gradual loss of the H_1 plasmid is seen when H_2 is the resident plasmid (Fig. 1A). When H_1 is the resident, however, some of the cells lose H_1, whereas the rest retain both plasmids (Fig. 1B). Both plasmids are maintained separately and can be recovered throughout the growth period.

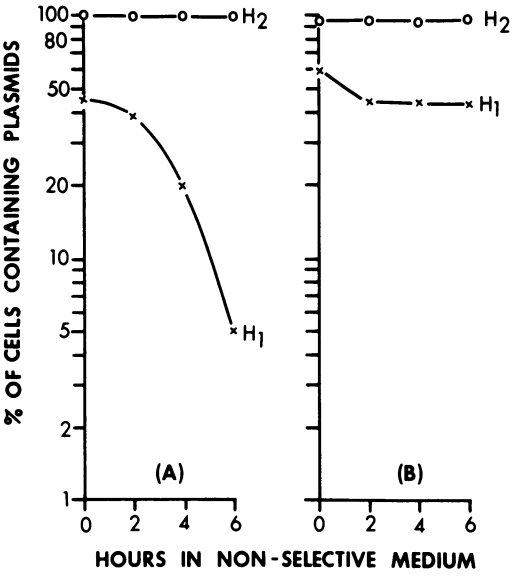

FIG. 1. *Kinetics of incompatibility between H_1 and H_2 plasmids. Transconjugant clones were prepared by selecting for both pRG1251-2 and pAS251-2-1 as described in footnote a of Table 5. Clone 1 (A) is substrain RG488; pAS251-2-1 (H_2) is the resident plasmid, and pRG1251-2 (H_1) is the incoming plasmid. Clone 2 (B) is RG176; pRG1251-2 is the resident plasmid, and pAS251-2-1 is the incoming plasmid. Cells from each clone were suspended in 1 ml of Penassay broth, grown at 37°C, and tested periodically for the presence of the plasmids.*

Incompatibility of bacterial plasmids has been shown to depend on the inhibition of replication of one of the plasmids (10, 15). The nonreplicating plasmid then becomes diluted out during subsequent cell growth and division (18). Models that may be used to explain incompatibility postulate either positive control, for which a replicational or segregational site is required (14), or negative control, for which identical or cross-reacting repressors are formed by incompatible plasmids (18). Recent work has favored the latter hypothesis (3, 22). H plasmids exhibit a type of "hierarchy" relationship, previously noted for F and ColV (16), which belong to the FI incompatibility group, for JR71 and R1 (7), which belong to group FII,

TABLE 5. *Colony tests plasmid, of incompatibility between plasmids of the H_1 and H_2 incompatibility groups after double-antibiotic selection* [a]

Transconjugant clone	Plasmid		% Daughter colonies containing:[b]		
	Incoming	Resident	Incoming plasmid only	Resident plasmid only	Both plasmids
1	pRG1251-2 (H_1)	pAS251-2-1 (H_2)	0	48	52
2	pAS251-2-1 (H_2)	pRG1251-2 (H_1)	40	4	56

[a] Transconjugant clones were prepared by selection with chloramphenicol and tetracycline. Single clones of substrain RG488 (clone 1) and RG176 (clone 2) were picked, diluted, and grown under nonselective conditions as described in the text. From each clone, 100 daughter colonies were tested for the loss of pRG1251-2 (ampicillin and tetracycline resistance) or pAS251-2 (chloramphenicol resistance).

[b] These values are the zero-time values for the plasmid segregation curves shown in Fig. 1.

and for plasmids of the I incompatibility group (6). The type of incompatibility seen between H subgroups can be best explained in terms of a negative control model for incompatibility. In the case of homo-incompatibility between members of the same subgroup, the phenomenon would depend on identical repressor molecules, so that antibiotic selection only determines which plasmid remains. Hetero-incompatibility between H_1 and H_2 could be accounted for by cross-reacting repressors: the H_2 repressor would bind more strongly to the H_1 operator site than the H_1 repressor would bind to the H_2 operator. Thus, replication of the H_1 plasmid would usually be suppressed, whereas H_2 replication would continue.

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