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Inhibition of F*lac* Transfer by the Fin$^+$ I-Like Plasmid R62

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F*lac* mutants have been isolated in *Escherichia coli* K-12 which carry dominant mutations resulting in insensitivity to transfer inhibition by the Fin$^+$ I-like plasmid R62. These mutants were still sensitive to transfer inhibition by the *fin*$^+$ F-like plasmid R100 and, conversely, F*lac traO*$^-$ and *traP*$^-$ mutants, which are insensitive to R100 inhibition, were still sensitive to R62. The sites of action of the two inhibition systems are therefore different. Furthermore, inhibition by R62, unlike R100, did not require an F-specified product. Like R100, R62 prevented transfer, pilus formation, and surface exclusion and, therefore, probably inhibits expression of the transfer operon *traA* through *traI*. However, R62 was different from R100 in inhibiting transfer of J-independent mutants, indicating that its effect on the transfer operon is probably direct rather than via *traJ*. This is consistent with the different sites of action of the two inhibition systems. None of the F*lac* mutants overproduced pili in the absence of R62, although one mutant differing from those described above showed increased levels of transfer and surface exclusion.

R62 is a sex factor producing I-like pili (6, 9) and possessing deoxyribonucleic acid sequences homologous to both I-like and group N plasmids (5). Unexpectedly, it was found to inhibit transfer and pilus production by an F factor when present in the same cell, i.e., to be Fin$^+$ (6, 9). However, the mechanism of transfer inhibition was different from that of *fin*$^+$ F-like plasmids, since R62 still inhibited transfer and pilus production by mutants of F-like plasmids insensitive to inhibition by the *fin*$^+$ product (6). Furthermore, mutants of F-like plasmids insensitive to inhibition by R62 were isolated and found to overproduce pili in the absence of the R factor.

In previous studies of F transfer inhibition by *fin*$^+$ F-like plasmids, we have shown that both the *fin*$^+$ product and the product of an F gene, *traP*, are required (3) and that the inhibitor acts at site *traO* to prevent synthesis or function of the F *traJ* product (4, 14). In this study we have extended our findings to R62 by showing that both *traP*$^-$ and *traO*$^-$ mutants of F*lac* are still sensitive to inhibition by R62. Furthermore, F*lac* mutants isolated as being insensitive to inhibition by R62 were still inhibited by the *fin*$^+$ F-like plasmid R100. All of these mutants except one carried dominant mutations presumed to be in the site of action of the R62 transfer inhibitor. However, none of them overproduced F pili in the absence of R62.

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MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* K-12 strains used as plasmid hosts are described in Table 1.

JCFLO is a wild-type F*lac* element, described by Achtman et al. (2). R62 was the generous gift of N. Datta.

Media. The media have been described previously (3, 4).

Bacterial matings. The techniques of Finnegan and Willetts (3, 4) for measuring the frequency of plasmid transfer, the level of surface exclusion, and the frequency of retransfer of F*lac* from newly infected cells were followed. ColIb^R recipient strains were used in matings since R62 determines production of colicin Ib (9).

Phage techniques. Sensitivities to the F-specific phages f1, Qβ, M12, and R17 were determined by spot tests and plaque assays as described by Achtman et al. (2).

Quantitative ^{32}P-labeled R17 adsorption to piliated cells was measured by adding an excess (10^4 physical particles per cell) to log-phase broth cultures (10^8 cells per ml) and allowing adsorption at 4 C for 40 min. The cells were then pelleted by centrifugation, resuspended in buffer, and counted to determine the amount of adsorbed radioactive phage. F$^-$ cultures were used as controls to correct for nonspecific trapping of phage in the cell pellet.

Mutagenesis. Treatment with ethyl methane sulfonate was carried out as described by Willetts (10). Treatment with *N*-methyl-*N*-nitroso-*N'*-ni-

TABLE 1. *Bacterial strains*[a]

Strain no.	His	Trp	Lys	Lac	T6	Str	Spc	Other markers
ED24	+	+	+	−	R	S	R	
ED26	−	−	−	−	R	R	S	ColIb^R
ED2197	+	+	+	−	R	S	R	ColIb^R Nal^R
ED2601	−	−	−	−	R	R	S	Fla$^-$
JC3272	−	−	−	−	R	R	S	
JC5455	−	−	+	−	R	S	R	
JC6255	+	−	+	−	S	S	S	Su$_I^+$
JC6256	+	−	+	−	S	S	S	

[a] The derivations of ED24, JC3272, JC5455, JC6255, and JC6256 are given by Finnegan and Willetts (3), ED26 is a spontaneous ColIb^R mutant of JC3272, ED2197 is a ColIb^R Nal^R derivative of ED24, and ED2601 is an ethyl methane sulfonate-induced Fla$^-$ mutant of JC3272.

trosoguanidine (NTG) was similar, except that the log-phase cells were suspended in 5 ml of 0.1 M citrate buffer (pH 5.5) and treated with 100 μg of nitrosoguanidine per ml for 30 min at 37 C.

Isolation of R62 inhibitor-insensitive Flac mutants. Cultures of JC6256 carrying CJFLO (together with R62 in later experiments) were treated with ethyl methane sulfonate or nitrosoguanidine. After dilution and overnight growth in broth, the mutagenized cultures were grown to 2×10^8 cells per ml, and 0.1 ml was mixed with 0.1 ml of an exponential culture of JC5455 carrying R62. After incubation at 37 C for 40 min, the mating was interrupted by treatment for 20 min with 0.2 ml of an ultraviolet-killed T6 lysate (10^{11} particles per ml). A 0.6-ml volume broth was then added, and the culture was incubated for a further 40 min to allow expression of the transfer genes. A 1.0-ml amount of JC3272 carrying R62 was added, and the mixture was incubated for 40 min. Appropriate dilutions were plated on medium selective for Lac$^+$ Tet^R [Str^R] progeny of JC3272. This procedure was designed to enrich for F*lac* mutants able to transfer at high frequency in the presence of R62.

The Lac$^+$ progeny clones were patched on nutrient plates, and their donor abilities were determined in replica-plate matings with ED24, selecting Lac$^+$ [His$^+$Trp$^+$Lys$^+$] progeny. Clones carrying JCFLO and R62 showed a low frequency of *lac*$^+$ transfer, and clones carrying a mutant JCFLO element insensitive to inhibition by R62 were detected by their high frequency of *lac*$^+$ transfer. Only one mutant was selected from each mutagenized culture, and each culture was started from a separate single colony of the JC6256 derivative.

RESULTS

Pilus production by R62-insensitive Flac mutants. Seven independent R62-insensitive F*lac* mutants, isolated by the techniques described above, were tested for the level of pilus production in the absence of R62. At first, this was done by measuring the number of ^{32}P-labeled R17 phage particles bound per cell: increased numbers of pili should be reflected in increased numbers of phage particles bound. However, no increases were found for cells carrying the R62-insensitive mutants; on the contrary, these cells bound fewer phages than cells carrying JCFLO (Table 2). The decrease in phage binding was small, except for cells carrying EDFL156.

To exclude the possibility that the mutants were producing increased numbers of pili with fewer R17 binding sites per pilus, the cells were also examined in the electron microscope, and the number of pili per cell was directly counted. To facilitate this, a flagella-minus (Fla$^-$) mutant, ED2601, was used as host strain. This confirmed that, except for EDFL156, the numbers of pili per cell produced by the mutants and by JCFLO were approximately the same (Table 2). Pili on cells carrying mutant plasmids were one-half to three-quarters as long, on average, as those seen on cells carrying JCFLO. Cells carrying EDFL156 had sixfold fewer pili per cell, accounting for the decrease in radioactive R17 phage binding.

Transfer properties of R100- and R62-insensitive Flac mutants. The donor abilities, F-specific phage sensitivities, and surface exclusion indexes of cells carrying JCFLO, either alone or in the presence of R100 or R62, were measured (Table 3). Both R factors inhibited all three wild-type phenotypes of transfer, pilus production, and surface exclusion.

If instead of JCFLO, an R100-insensitive F*lac*

TABLE 2. *Pilus production by R62-insensitive mutants*

F*lac* element[a]	Relative plating efficiency of R17 phage[b]	Relative attachment of ^{32}P-R17 phage[b]	Level of piliation seen by electron microscopy[c]
JCFLO	100	100	103
WPFL36	93	77	85
WPFL39	85	70	98
EDFL152	83	73	90
EDFL153	81	63	80
EDFL154	82	65	80
EDFL155	76	79	92
EDFL156	86	18	16

[a] Host strain was ED2601.

[b] Mean values from three sets of determinations. The JCFLO derivative of ED2601 adsorbed 600 to 700 physical particles per cell under the conditions of the assay.

[c] Pili per 100 cells determined as described by Paranchych et al. (7). Mean values from two sets of determinations are given.

TABLE 3. *Properties of the inhibitor-insensitive mutants*

F*lac* element[a]	Mutation	Donor ability[b]			Phage sensitivity[c]			Surface exclusion[d]		
		—	R100	R62	—	R100	R62	—	R100	R62
JCFLO		135	0.06	1.1	S	R	R	300	6	6
EDFL51	*traP301*	150	160	0.9	S	S	R	300	250	6
EDFL67	*traO304*	120	130	0.5	S	S	R	500	550	6
WPFL36	*tra-317*	80	0.3	75	S	R	S	550	10	180
WPFL39	*tra-312*	125	0.2	95	S	R	S	490	7	190
EDFL155	*tra-316*	70	0.02	70	S	R	S	350	8	170
EDFL156	*tra-318*	200	0.3	25	S	R	S	1,530	70	130

[a] EDFL51 and EDFL67 were described by Finnegan and Willetts (3). *tra-312*, *tra-316*, *tra-317*, and *tra-318* are mutations giving insensitivity to inhibition by R62. Results are given for derivatives carrying no R factor, or R100, or R62.

[b] Donor strains were derivatives of JC6255 or JC6256 (for JCFLO, EDFL155, and EDFL156), and the recipient strain was ED26. The numbers represent the Lac$^+$ [Str^R] progeny per 100 donor cells obtained after a 30-min mating. In all cases, R100 and R62 transferred at frequencies of about 0.4 and 0.04%, respectively.

[c] Spot tests on the JC6255 or JC6256 derivatives, using f1, Qβ, M12, and R17. All phages gave similar results, except that the strain carrying R62 and EDFL156 gave turbid spots with the ribonucleic acid phages (Qβ, M12, and R17).

[d] Numbers represent surface exclusion indexes, measured in matings between the Hfr strain KL98 and derivatives of JC3272.

mutant was used, none of the three properties was affected by R100 (3), but all were still inhibited by R62 (Table 3). This was true for mutations in the site of action of the R100 transfer inhibitor (*traO*) or in the F-specified component of the transfer inhibitor (*traP*). Similar results were obtained, but the data are not given, for a second *traO* mutant (EDFL68, carrying *traO305*) and a second *traP* mutant (EDFL55, carrying *traP303*). These results show that the sites of action of the R62 and R100 transfer inhibitors are probably different and that the F *traP* product (P_F) is not required for inhibition by R62.

Reciprocal results were obtained when the R62-insensitive F*lac* mutants were tested; transfer, pilus production, and surface exclusion by these mutants were not inhibited by R62, but were still inhibited by R100 (Table 3). Except for EDFL156, similar results were obtained for all the R62-insensitive mutants, and data for three of these are therefore omitted. These results again suggest that the mechanisms of transfer inhibition by R62 and by R100 are different.

EDFL156 was unusual in showing significantly higher levels of donor ability and surface exclusion than JCFLO; however, this was not accompanied by overtly high levels of F pili per cell, rather the reverse. Furthermore, both donor ability and surface exclusion were reduced about 10-fold by R62, so that R62 insensitivity was only partial. Given the high starting levels, reductions of transfer and surface exclusion by R100 were approximately the same as for JCFLO, demonstrating that EDFL156 is still sensitive to inhibition by R100. This unusual mutant will be discussed further.

Dominance of the R62-insensitive mutations. Dominance was measured in transient populations of heterozygous cells as described previously (3, 4). Briefly, the F*lac* mutant was transferred from JC6256 (T6^S) to JC5455 (T6^{R}Str^S) carrying both R62 and F*his*. After T6 killing, transfer of F*lac* from the resultant heterozygous cells to ED26 (Lac$^-$T6^{R}Str^R) was measured.

Preliminary experiments with wild-type F*lac* showed that its retransfer from cells carrying R62 alone was inhibited (Table 4). This behavior is different from that of cells carrying R100 in which F transfer inhibition is not expressed for several hours (3, 11). It provides further evidence that transfer inhibition by R62 does not require P_F or indeed any other F product.

Retransfer of the R62-insensitive F*lac* mutants (except for EDFL156) took place at similar frequencies from intermediate cells carrying no plasmids, R62 alone, or both R62 and F*his* (Table 4). The mutant phenotype was, therefore, expressed even in the presence of the wild-type allele carried by F*his*, and these mutants must carry dominant mutations which are presumably in the site of action of the R62 transfer inhibitor. Complementation tests between dominant mutations are not possible, but based upon the phenotypic similarities these six R62 inhibitor-insensitive F*lac* mutants are all considered to carry mutations at a site designated *traQ*.

TABLE 4. *Retransfer of R62-insensitive Flac mutants*[a]

Flac element	Mutation	Plasmids in intermediate strain		
		—	R62	R62 and *Fhis*
JCFLO		61	0.06	0.09
WPFL36	*traQ317*	38	28	12
WPFL39	*traQ312*	43	39	16
EDFL152	*traQ313*	49	48	39
EDFL153	*traQ314*	68	48	20
EDFL154	*traQ315*	72	48	36
EDFL155	*traQ316*	64	53	38
EDFL156	*tra-318*	88	0.09	0.09

[a] The *Flac* element was transferred from JC6255 or JC6256 to the intermediate strain (JC5455) carrying the plasmids indicated. After killing the JC6256 donor with T6, the frequency of *Flac* retransfer to ED26 (per 100 intermediate cells which had received it) was measured. Transfer of R62 from the intermediate strains took place at frequencies of 0.001 to 0.01%. The frequency of transfer of the *Fhis* element, when present in the intermediate strain, was always closely similar to that of the *Flac* element (3, 4).

Unlike the six *traQ*$^-$ mutants, retransfer of EDFL156 from the intermediate strain carrying R62 took place at the same low frequency as that of JCFLO itself (Table 4). The R62 inhibitor insensitivity of EDFL156 was therefore not expressed under these conditions, and there must be a delay before this occurs. The dominance test was consequently not interpretable. This result further emphasizes the difference between *tra-318* and the *traQ* mutations.

That no recessive R62-insensitive mutations were found among the seven independent *Flac* mutants tested again indicates that no F product is required for transfer inhibition by R62.

R62 and "J-independent" Flac mutants. The *traJ* product is normally required for synthesis of all the other *tra* products that are directly involved in pilus formation, surface exclusion, and deoxyribonucleic acid transfer (4, 12). Achtman (1) isolated a series of partial Tra$^+$ revertants of *Flac traJ90* that transferred with frequencies of 0.05 to 1% from a Su$^-$ strain, even though they still carried the amber *traJ90* mutation. Their secondary J-independent mutations are, consequently, thought to allow partial expression of the transfer genes in the absence of the *traJ* product. This partial expression was not inhibited by R100, presumably because the R100 transfer inhibitor acts by preventing synthesis of the *traJ* product (1, 4, 11).

If the R62 transfer inhibitor also acts via *traJ*, transfer by J-independent mutants should similarly be insensitive to inhibition by R62, and this was therefore tested. The results (Table 5, columns 2 to 4) confirm that JCFL119, JCFL129, and JCFL130 carried an amber *traJ* mutation, together with J-independent mutations, allowing low levels of transfer from a Su$^-$ host that were unaffected by R100. R62, however, reduced transfer of the mutants by about 20-fold (Table 5, column 5). This suggests that R62 prevents expression of the transfer genes directly and not via *traJ*.

DISCUSSION

Our results confirm and extend the finding of Meynell (6) that transfer inhibition by R62 differs from that by *fin*$^+$ F-like plasmids such as R100. In particular, *Flac* mutants insensitive to inhibition by the R100 transfer inhibitor because of dominant mutations in its site of action, were still sensitive to inhibition by the R62 transfer inhibitor, and vice versa. Furthermore, inhibition of *Flac* transfer by R62 required niether the F *traP* product nor (probably) any other F product.

R. Helmuth and M. Achtman (manuscript in preparation) have recently shown that all of the *tra* genes except *traJ* are present in a single operon (the transfer operon). *traS*, the gene(s) responsible for surface exclusion, is also a part of this operon (12, 14). Therefore, the inhibition of transfer, pilus formation, and surface exclusion produced by both R100 and R62 indicates that they inhibit expression of the transfer operon. In the case of R100, this is achieved

TABLE 5. *Transfer inhibition of J-independent Flac mutants*[a]

Flac element	No. of progeny			
	Su_I^+	Su$^-$		
		—	R100	R62
JCFLO	58	71	0.038	0.82
JCFL119	100	1.3	1.6	0.038
JCFL129	49	0.044	0.079	0.0037
JCFL130	34	0.059	0.098	0.0026

[a] JCFL119, JCFL129, and JCFL130 are J-independent mutants (1). The figures represent the number of Lac$^+$ [Nal^R] progeny per 100 donor cells obtained using a Su_I^+ (JC6255) or a Su$^-$ (JC6256) donor strain carrying the R factor indicated. The recipient strain was ED2197. Since the transfer levels were sometimes relatively low, a Nal^R rather than Str^R contraselection was used to prevent interference by relatively frequent high level Str^R mutants of cells carrying R100 or R62 (8).

indirectly by preventing synthesis of the *traJ* product that is normally required for expression of the operon (4, 11, 14). Transfer inhibition by R62, however, must have a different mechanism since the site of action is different and transfer of J-independent mutants is inhibited. Both of these observations can be explained by the hypothesis that the R62 transfer inhibitor directly prevents the expression of the transfer operon otherwise permitted by the presence of the *traJ* product or a J-independent mutation.

EDFL156 differed from the other R62-insensitive mutants in several ways. Its increased levels of transfer and surface exclusion might be explained by increased expression of the transfer operon, but the expected simultaneous overproduction of pili was not apparent. This latter point is being investigated to determine whether any secondary effect is responsible. If so, there may be similarities between this mutant and the R62-insensitive pilus-overproducing mutants of R538-1*drd* and "F/R1-19" described by Meynell (6). Since EDFL156 (but not the *traQ*$^-$ mutants) showed delayed expression of its R62 insensitivity, differences in mutant selection procedures would be expected to influence the type of R62-insensitive mutant obtained. This (together with any intrinsic differences between the plasmids used) might explain why the types of R62-insensitive mutants isolated by us and by Meynell (6) were different.

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