

## Biosynthesis of the Outer Membrane Receptor for Vitamin B<sub>12</sub>, E Colicins, and Bacteriophage BF23 by *Escherichia coli*: Kinetics of Phenotypic Expression After the Introduction of *bfe*<sup>+</sup> and *bfe* Alleles

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The *bfe* locus codes for the cell surface receptor for vitamin B<sub>12</sub>, the E colicins, and bacteriophage BF23 in the *Escherichia coli* outer membrane. When the *bfe*<sup>+</sup> allele, which is closely linked to the *argH* locus, was introduced into an *argH bfe* recipient by conjugation, *arg*<sup>+</sup> recombinant cells rapidly and simultaneously acquired sensitivity to colicin E3 and phage BF23. In the reciprocal experiment introducing *bfe* into an *argH bfe*<sup>+</sup> recipient, it was found that colicin E3-resistant, *arg*<sup>+</sup> cells began to appear shortly after the *arg*<sup>+</sup> recombinant population began to divide. This was far earlier than would have been predicted on the basis of 220 receptors per haploid cell. Moreover, there was a lag between the appearance of colicin resistance and the appearance of resistance to killing by phage BF23, and hence a period of time during which some *arg*<sup>+</sup> recombinant cells were sensitive to the phage but resistant to the colicin. Colicin E3 added to cells during this period of time protected against phage killing, indicating that the colicin-resistant cells still had receptors capable of binding colicin on their surface. The modification of the phenotypic expression of colicin and phage resistance by inhibitors of deoxyribonucleic acid, ribonucleic acid, and protein synthesis was also investigated. The results obtained indicate that the receptor protein coded for by the *bfe* locus can exist on the cell surface in several different functional states.

Certain proteins in the outer membrane of *Escherichia coli* serve as the cell surface receptors for bacteriophages and colicins (22). These proteins undoubtedly perform some function useful to the bacterial cell, or their presence in the outer membrane should have long ago been selected against. Recent studies have shown that certain colicin and phage receptors are specific components of various nutrient transport systems. For example, Szmecman and Hofnung (31) have demonstrated that the phage λ receptor is also involved in the transport of maltose and the higher maltodextrins. The *tonA* gene product, the receptor for phages T1 and φ80 and colicin M, also binds ferric-ferriochrome and is required for its utilization (11, 32).

DiMasi et al. (7) and Bradbeer et al. (3) have demonstrated that the *bfe*<sup>+</sup> gene product, which serves as the outer membrane receptor for phage BF23 and the E colicins (4, 15), is also the receptor for vitamin B<sub>12</sub>. Vitamin B<sub>12</sub> auxotrophs which are resistant to colicin E3 require greatly elevated levels of B<sub>12</sub> in the culture

medium for normal growth (17). Sabet and Schnaitman (28) purified the colicin E receptor and showed it to be an outer membrane protein of 60,000 daltons, present in approximately 220 copies per cell.

We have chosen to study the functional heterogeneity of the *bfe*<sup>+</sup> product in some detail in hopes of increasing our knowledge of the structure and function of the outer membrane of *E. coli*. By comparing the kinetics of appearance of colicin E3 and phage BF23 sensitivity or resistance after conjugal entry of either the wild-type or mutant allele, we have demonstrated that the *bfe*<sup>+</sup> product can apparently exist in several different "states" on the cell surface. These states can be influenced by the addition of certain antibiotics and the state of the receptor can determine the killing ability of adsorbed colicin or phage particles.

In an accompanying paper by Kadner and Bassford (16), a different experimental approach has been employed to study the relationship between vitamin B<sub>12</sub> and colicin E uptake in *E. coli*.

## MATERIALS AND METHODS

**Media.** AF (arginine-free) medium is minimal medium A of Davis and Mingioli (6) supplemented with a mixture of amino acids, purines, pyrimidines, and vitamins, as described by Novick and Maas (27), and includes glucose (0.5%) and streptomycin (100  $\mu$ g/ml). Luria broth without glucose was used for all matings. Cells were grown at 37°C with vigorous aeration. Spectinomycin, chloramphenicol, and nalidixic acid (concentrations as indicated) were added as aqueous solutions. A rifampin stock solution (50 mg/ml) was made up in methanol and diluted with AF medium. Agar in plates was used at a final concentration of 2.0%. A soft agar was medium A containing 0.7% agar. Most chemicals were obtained from the Sigma Chemical Co.

**Bacterial strains.** KL209 is Hfr *sup-53 malB12* (23) and was obtained from J. Miller. RK24 is the same as KL209 but *bfe*. RK4113 is F<sup>-</sup> *leu proC lysA metE argH tonA strA* and was derived from RK4101 (17). RK4116 is the same as RK4113 but *bfe*. Both RK24 and RK4116 were obtained by selecting for spontaneous resistance to bacteriophage BF23. During the course of this study, several independently derived *bfe* derivatives of KL209 and RK4113 were employed, each time with identical results.

**Preparation and standardization of colicins and bacteriophage.** Colicins E2 and E3 were prepared as described in the accompanying paper (16) and diluted as previously described (28). The colicin E3 stock preparation contained 10<sup>6</sup> killing units as defined by Sabet and Schnaitman (28). Bacteriophage BF23 was obtained from R. Benzinger and prepared from broth lysates by the method of Yamamoto et al. (33). L broth was used for phage adsorption.

Colicin E3 or phage BF23 sensitivity was determined by mixing 0.1 ml of bacterial cells in AF medium with 0.1 ml of a dilution of either colicin or phage and incubating for 5 min at 37°C. Adsorption was terminated by the addition of 0.1 ml of 15  $\mu$ M B<sub>12</sub> followed by Vortex mixing. These samples were then mixed with 3 ml of A soft agar containing 5  $\mu$ M B<sub>12</sub> and poured onto AF agar plates. Dilutions, when required, were made with AF medium containing 5  $\mu$ M B<sub>12</sub> after the 5-min adsorption period with colicin or phage.

To use such short incubation periods with phage or colicin, it was necessary to use the highest possible multiplicity of infection of these agents, and to standardize the titers of phage and colicin so that identical killing was obtained with both agents under these conditions. In practice, it was found that a 1:125 dilution of the colicin E3 stock preparation was the highest concentration of colicin which could be employed. Higher concentrations of colicin resulted in significant killing of colicin-resistant cells. It was found that a phage BF23 suspension containing  $2 \times 10^{11}$  plaque-forming units/ml yielded the same extent of killing as a 1:125 dilution of the colicin stock, and this concentration of phage had no effect on resistant cells. A minimum multiplicity of infection of 10,000:1 of colicin or phage to cells resulted from the use of these concentrations in all the experiments described in this paper.

**Conjugation.** The Hfr and F<sup>-</sup> strains used in these experiments were grown in L broth to a density of approximately  $3 \times 10^8$  cells/ml. Strains were then mixed in a ratio of 5 Hfr cells to 1 F<sup>-</sup> cell and incubated without agitation at 37°C. After a 25-min mating period, conjugation was terminated by the use of a vibratory shaker (23) followed by immediate 1:500 dilution into AF medium. Under these conditions, approximately 1 to 5% of the recipient cells were *arg*<sup>+</sup> recombinants.

## RESULTS

**Kinetics of expression of Bfe<sup>+</sup> in exconjugants.** Buxton (4) had shown that Bfe<sup>+</sup> was dominant over Bfe<sup>-</sup> in merodiploids. For the present study, it was first of interest to examine the kinetics of expression of the *bfe*<sup>-</sup> allele in exconjugants. Strain KL209 is an Hfr that introduces the *bfe* locus as an early marker (23). After a mating between strain KL209 and RK4116 (F<sup>-</sup>), one finds that the donor *bfe*<sup>+</sup> marker is inherited by 93% of the *argH*<sup>+</sup> recombinants. To determine the actual time of acquisition of the Bfe<sup>+</sup> phenotype among the *arg*<sup>+</sup> recombinants, one can take advantage of an observation by DiMasi et al. (7) that vitamin B<sub>12</sub> protects sensitive cells against the lethal adsorption of colicin E3. B<sub>12</sub> also totally inhibits the lethal adsorption of phage BF23 (3). B<sub>12</sub> does not prevent the killing of cells to which the colicin or phage has been previously adsorbed. Upon the addition of B<sub>12</sub> to a mixture of sensitive cells and colicin E3 or sensitive cells and phage BF23, the lethal adsorption of the colicin or phage is immediately terminated.

Figure 1 shows the time of acquisition of the Bfe<sup>+</sup> phenotype by the recipient cells. Significant numbers of *arg*<sup>+</sup> recombinants were recorded in samples taken 10 min after the onset of mating, and this number continued to increase for the next 15 min at which time the mating mix was interrupted and diluted. At 25 min, almost none of the cells that had received the *argH*<sup>+</sup> gene were killed by either colicin or phage. Shortly thereafter, however, the recombinant population began to express the Bfe<sup>+</sup> phenotype, as measured by a decline in the number of recombinants after a period of colicin or phage adsorption terminated by the addition of B<sub>12</sub>. Note that under the conditions employed in the experiment, sensitivity to both colicin E3 and phage BF23 appeared concomitantly. By 70 min 97% of the *arg*<sup>+</sup> population had expressed the Bfe<sup>+</sup> phenotype. Although only 93% of the *arg*<sup>+</sup> cells would have been expected to inherit the *bfe*<sup>+</sup> allele, all of the recombinants should have received the gene, since the *bfe* locus is donated by strain KL209 prior to *argH*. These results would suggest, therefore, that the *bfe*<sup>+</sup>

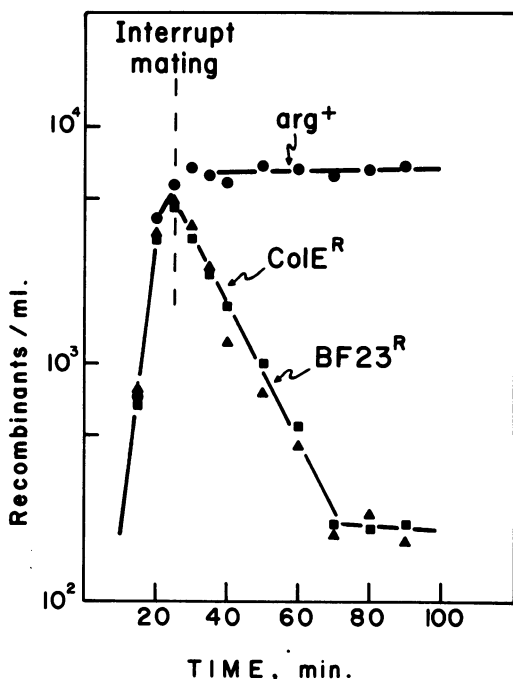


FIG. 1. Phenotypic expression of sensitivity to colicin E3 and phage BF23 in a resistant recipient strain (RK4116) after conjugation with a sensitive donor strain (KL209). Donor and recipient strains were mixed at zero time, and mating was terminated 25 min later. After the cells were diluted 1:500 in AF medium, the culture was placed in a rotary shaker at 37°C. The number of *arg*<sup>+</sup> recombinants present in the culture at any given time was determined by viable counts on AF agar plates. Mating conditions and the determination of colicin and phage sensitivity are described in Materials and Methods. Symbols: Total *arg*<sup>+</sup> recombinants (●); colicin-resistant, *arg*<sup>+</sup> recombinants (▲); phage BF23-resistant, *arg*<sup>+</sup> recombinants (■). Data points for colicin- and phage-resistant recombinants correspond to the time at which colicin or phage adsorption was terminated by *B*<sub>12</sub> addition. Data points prior to 25 min were obtained by prematurely interrupting parallel cultures in the presence of spectinomycin (100 µg/ml) to arrest further protein synthesis in the recipients.

allele does not have to recombine to be expressed, as was also observed for the gene *tsx*<sup>+</sup> (21). If this is the case, then it is not known why 100% of the recipient population did not become at least transiently colicin and phage sensitive, but all of the colicin- and phage-resistant *arg*<sup>+</sup> recombinants observed later than 70 min after mating were genotypically *bfe*.

It should be noted that 5 µM *B*<sub>12</sub> does provide 100% protection against the lethal adsorption of phage BF23 in liquid medium. Under identical conditions, *B*<sub>12</sub> provided only about 60 to 70%

protection against the lethal adsorption of colicin E3. This degree of protection was not increased by going to higher concentrations of *B*<sub>12</sub>. However, it was found that once the cells were embedded in A soft agar, 5 µM *B*<sub>12</sub> would completely protect against the E colicins, as demonstrated by the early data points in Fig. 1. Thus, recombinant cells expressing the *Bfe*<sup>+</sup> phenotype after plating in soft agar containing 5 µM *B*<sub>12</sub> were protected and subsequently scored.

**Acquisition of the *Bfe*<sup>-</sup> phenotype.** In the next experiment, *argH*<sup>+</sup> *bfe* was introduced into an *argH* *bfe*<sup>+</sup> recipient. Resistance to colicin E3 and phage BF23, being recessive to sensitivity, cannot be expressed in exconjugants until the following conditions are met: (i) the wild-type allele coding for fully functional receptors is no longer being transcribed; and (ii), functional receptors are no longer present on the surface of the cell. It is impossible to determine when the first condition is satisfied. Presumably, the *bfe*<sup>+</sup> allele is lost as a consequence of the recombination event. The loss of functional receptors can be determined by the acquisition of resistance to either colicin E3 or phage BF23. There are approximately 220 *B*<sub>12</sub> receptors per haploid cell (28). If, for example, all these receptors remain fully functional and are equally distributed to daughter cells during subsequent cell divisions, resistant recombinants would not appear until seven to eight generations after the loss of the *bfe*<sup>+</sup> gene.

The results of an experiment in which strain RK24 (*bfe*) was mated with strain RK4113 (*argH* *bfe*<sup>+</sup>) and the recombinants were allowed to grow out are presented in Fig. 2. There was a characteristic 2-h lag after the interruption of mating before the *arg*<sup>+</sup> recombinants began to divide (12). During this period, all the recombinant cells remained sensitive to both colicin E3 and phage BF23. Beginning approximately 120 min after mating, the recombinants resumed logarithmic growth with a doubling time of 35 min. The appearance of resistance to colicin E3 and phage BF23 followed different kinetics than that shown in Fig. 1. Colicin-resistant recombinants began to appear 150 min after mating, shortly after the recombinant population began to divide. By comparison, there was a considerable lag in the appearance of phage-resistant recombinant cells. For example, at 210 min after mating, there was nearly a 10-fold difference in the number of colicin-resistant and phage-resistant recombinants. It was not until almost 7 h after mating that all the recombinant cells acquiring the *bfe* allele expressed resistance to both the colicin and the

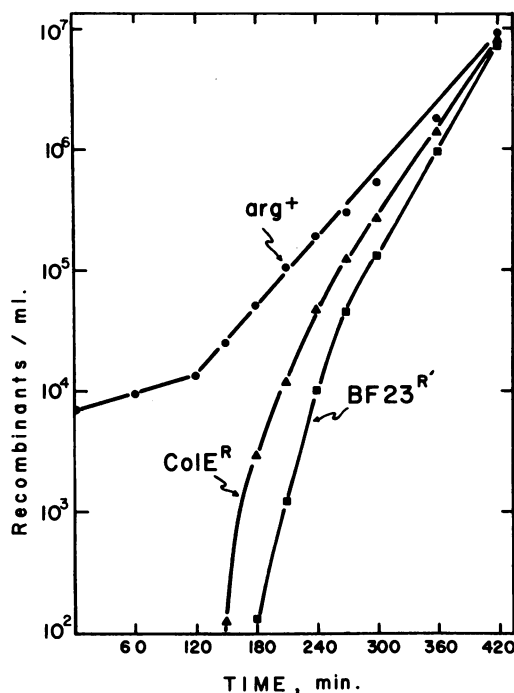


FIG. 2. Phenotypic expression of resistance to colicin E3 and phage BF23 in a sensitive recipient strain (RK4113) after conjugation with a resistant donor strain (RK24). Zero time indicates the time at which mating was terminated and the culture was diluted. Experimental conditions are described in Materials and Methods and in Fig. 1. Symbols: Total  $arg^+$  recombinants ( $\bullet$ ); colicin E3-resistant,  $arg^+$  recombinants ( $\Delta$ ); phage BF23-resistant,  $arg^+$  recombinants ( $\blacksquare$ ). Data points for colicin and phage resistance correspond to the time at which the cells were exposed to the agent.

phage. During this period, the recombinant population would have divided through approximately eight generations and, assuming 100% conservation of receptor molecules present prior to the onset of cell division, the recombinant cells would still average less than one receptor molecule on their surface.

Colicin tolerance precedes colicin resistance. If colicin E3 was still capable of adsorbing to that population of recombinant cells which was phenotypically colicin resistant and phage sensitive, then one should be able to protect these cells against the lethal adsorption of phage BF23 if the cells were first exposed to the colicin. In an experiment similar to the one described above, samples were removed from the culture at various times after mating and the number of colicin- and phage-resistant recombinants in the population was determined (Table 1). Additional samples were treated in

the following manner: (i) cells were pretreated with the standard dose of colicin E3 for 120 s prior to the addition of phage BF23; and (ii) cells were simultaneously exposed to an equal mixture of colicin and phage. If cells were first treated with colicin E3, the percentage of phage-resistant cells in the population equaled the percentage of colicin-resistant cells at each time point tested. When the cells were simultaneously treated with a mixture of colicin and phage, an intermediate value was obtained. Thus, it would appear that the colicin-resistant, phage-sensitive recombinant cells were colicin insensitive despite the presence of intact receptors on their cell surface which were capable of binding colicin molecules. This is a situation exactly identical to that found in colicin-tolerant mutants (24).

Effect of nalidixic acid on the phenotypic expression of colicin and phage resistance. If cell division is a requirement for the phenotypic

TABLE 1. Protection of  $arg^+$  recombinant cells against phage BF23 killing by colicin E3

Sample and treatment:	No. of recombinant cells		
	180 <sup>a</sup>	210	240
Controls:			
Total $arg^+$ recombinants	$3.4 \times 10^4$	$6.0 \times 10^4$	$1.2 \times 10^5$
$arg^+$ recombinants that were E3 resistant	$2.4 \times 10^3$	$8.2 \times 10^3$	$2.6 \times 10^4$
$arg^+$ recombinants that were BF23 resistant	$1.4 \times 10^3$	$9.0 \times 10^3$	$4.7 \times 10^3$
% of E3-resistant cells that were also BF23 resistant	5.6	10.9	18.0
Sample treated with E3 prior to the addition of phage: <sup>b</sup>			
$arg^+$ recombinants that were BF23 resistant	$2.9 \times 10^3$	$8.3 \times 10^3$	$2.6 \times 10^4$
% of E3-resistant cells that were also BF23 resistant	100	100	100
Sample treated with E3 and BF23 simultaneously: <sup>c</sup>			
$arg^+$ recombinants that were BF23-resistant	$1.5 \times 10^3$	$5.9 \times 10^3$	$2.2 \times 10^4$
% of E3-resistant cells that were BF23 resistant	62	72	84

<sup>a</sup> Minutes after mating.

<sup>b</sup> Cells were incubated with a standard dose of colicin E3 for 120 s prior to addition of phage BF23. Colicin diluent had no effect on phage BF23 adsorption.

<sup>c</sup> Cells were simultaneously treated with a standard dose of both colicin E3 and phage BF23.

expression of resistance to colicin E3 and phage BF23 in the recombinant population, then by inhibiting cell division one should also inhibit the further appearance of resistant recombinants. Nalidixic acid is an inhibitor of deoxyribonucleic acid synthesis in *E. coli* at concentrations as low as 1  $\mu\text{g/ml}$  (10). Since deoxyribonucleic acid synthesis apparently provides the signal for septum formation and subsequent cell division (14), the effect of nalidixic acid on the *arg*<sup>+</sup> recombinant population was investigated.

Although nalidixic acid is reported to have little effect on overall protein synthesis in *E. coli*, it has been shown to selectively inhibit the synthesis of certain proteins, including the receptor for phage  $\lambda$  (30). Strain KL209 was mated with strain RK4116. Five minutes after the termination of mating, nalidixic acid was added to the recombinant culture (Fig. 3). It was found that neither concentration of nalidixic acid used in the experiment had any effect

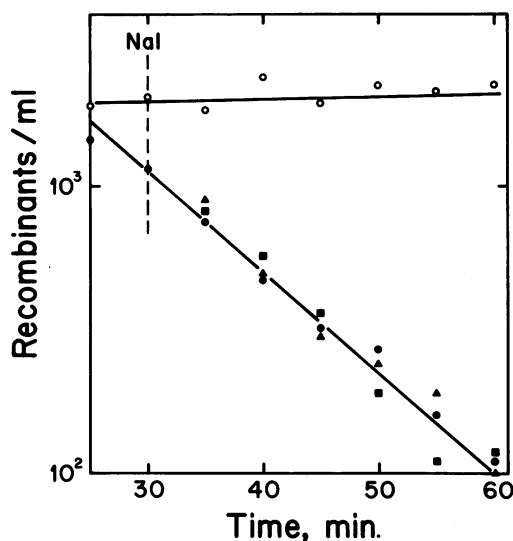


FIG. 3. Effect of nalidixic acid on the phenotypic expression of sensitivity to phage BF23 in a resistant recipient strain (RK4116) after conjugation with a sensitive donor strain (KL209). Experimental conditions are the same as Fig. 1. Five minutes after the termination of mating, the culture was divided into three portions: one control and one for each of the two different inhibitor concentrations employed. Symbols: Total *arg*<sup>+</sup> recombinants, no addition (○); BF23-resistant, *arg*<sup>+</sup> recombinants, no addition (●); BF23-resistant, *arg*<sup>+</sup> recombinants, 10  $\mu\text{g}$  of nalidixic acid per ml (▲); BF23-resistant, *arg*<sup>+</sup> recombinants, 50  $\mu\text{g}$  of nalidixic acid per ml (■). It should be noted that after 30 min in the presence of 50  $\mu\text{g}$  of nalidixic acid per ml, there was about a 30% decrease in the total number of *arg*<sup>+</sup> recombinants (not shown).

on the expression of the *bfe*<sup>+</sup> allele in the recipients, as determined by the acquisition of sensitivity to, in this case, phage BF23.

The effect of nalidixic acid on the expression of colicin E3 and phage BF23 resistance in ex-conjugant cells was then studied. Nalidixic acid was added to the recombinant culture 180 min after the mating of strain RK24 with strain RK4113 (Fig. 4). A final concentration of 1.5  $\mu\text{g}$  of nalidixic acid per ml was used, because it was found that higher concentrations resulted in a marked decrease in cell viability after a 30-min incubation with the inhibitor. With this concentration, the number of *arg*<sup>+</sup> recombinants did not stop increasing until approximately 60 min after the addition of the inhibitor. However, the number of colicin- and phage-resistant recombinants continued to increase. For example, the percentage of phenotypically colicin-resistant cells in the population increased from 17.2 to 32% over a 30-min period

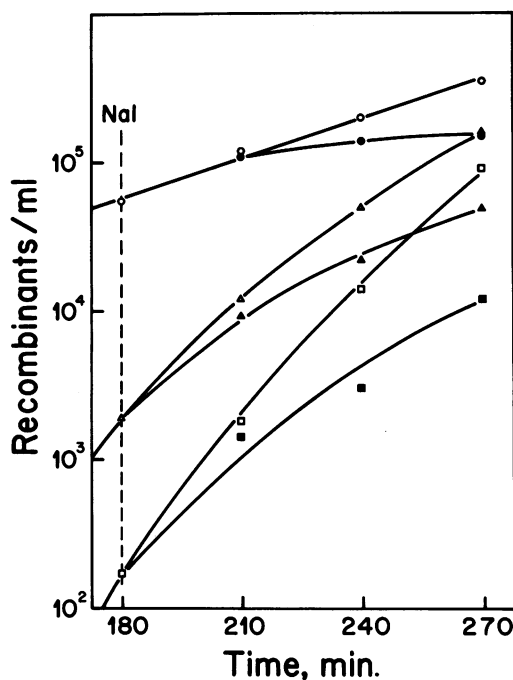


FIG. 4. Effect of nalidixic acid on the phenotypic expression of resistance to colicin E3 and phage BF23 in a sensitive recipient strain (RK4113) after conjugation with a resistant donor strain (RK24). Experimental conditions are the same as Fig. 2. Symbols: Total *arg*<sup>+</sup> recombinants (○); colicin E3-resistant, *arg*<sup>+</sup> recombinants (Δ); phage BF23-resistant, *arg*<sup>+</sup> recombinants (□); open symbols, a control culture; filled symbols, a culture to which nalidixic acid (1.5  $\mu\text{g/ml}$ ) was added 180 min after the termination of mating.

(240 to 270 min after mating) in which no increase in the total number of recombinants was observed.

**Effects of inhibitors of protein synthesis.** The antibiotic spectinomycin inhibits protein synthesis in *E. coli*, and at a concentration of 100  $\mu\text{g/ml}$  it is not bactericidal. When spectinomycin was added to the recombinant culture 210 min after the mating of strain RK24 with strain RK4113, the cells continued to divide through approximately an additional half generation (Fig. 5). Upon the addition of the antibiotic, the number of phenotypically colicin-resistant cells immediately leveled off. The percentage of colicin-resistant cells decreased from a value of 16.6% 210 min after mating to 11.2% 15 min after spectinomycin addition. This is further evidence that cell division is not the sole requirement for the expression of colicin resistance, i.e., colicin tolerance. The number of phage-resistant recombinants continued to increase in the presence of the antibiotic until it just about equaled the number of colicin-

resistant recombinants after a 45-min incubation with spectinomycin.

Chloramphenicol is also used as a reversible inhibitor of protein synthesis in *E. coli*. When chloramphenicol was added 210 min after mating, the total number of *arg*<sup>+</sup> recombinants continued to increase for approximately 30 min, after which there was a rapid decline in cell viability (Fig. 6). (A 30-min incubation with chloramphenicol at this concentration has been reported to affect the viability of *E. coli* [9].) As was previously observed for spectinomycin, the number of phage-resistant recombinants continued to increase for some time in the presence of chloramphenicol. However, the actual number of phenotypically colicin-resistant recombinants began to decrease immediately upon the addition of chloramphenicol and continued to decrease for the entire 45-min course of this experiment.

**Effect of rifampin.** The addition of rifampin 210 min after mating resulted in a gradual decline in the total number of *arg*<sup>+</sup> recombi-

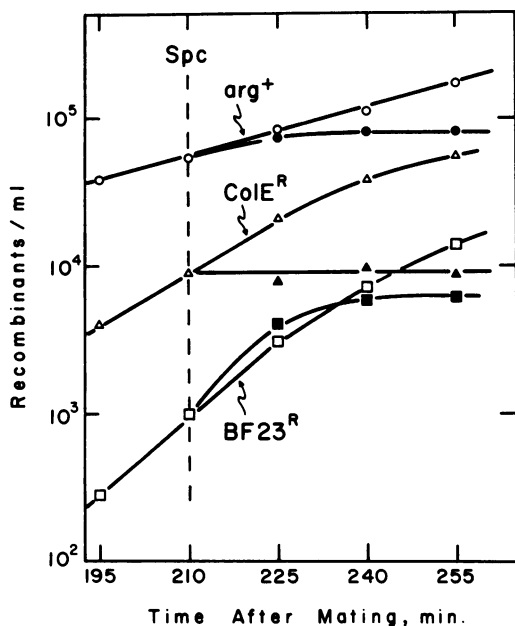


FIG. 5. Effect of spectinomycin on the phenotypic expression of resistance to colicin E3 and phage BF23 in a sensitive recipient strain (RK4113) after conjugation with a resistant donor strain (RK24). Experimental conditions are the same as Fig. 2. Symbols: Total *arg*<sup>+</sup> recombinants (○); colicin E3-resistant, *arg*<sup>+</sup> recombinants (△); phage BF23-resistant, *arg*<sup>+</sup> recombinants (□); open symbols, a control culture; filled symbols, a culture to which spectinomycin (100  $\mu\text{g/ml}$ ) was added 210 min after termination of mating.

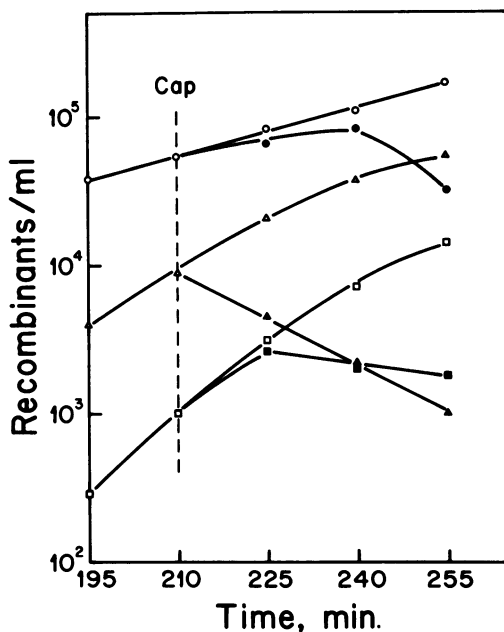


FIG. 6. Effect of chloramphenicol on the phenotypic expression of resistance to colicin E3 and phage BF23 in a sensitive recipient strain (RK4113) after conjugation with a resistant donor strain (RK24). Experimental conditions are the same as Fig. 2. Symbols: Total *arg*<sup>+</sup> recombinants (○); colicin E3-resistant, *arg*<sup>+</sup> recombinants (△); phage BF23-resistant, *arg*<sup>+</sup> recombinants (□); open symbols, a control culture; filled symbols, a culture to which chloramphenicol (100  $\mu\text{g/ml}$ ) was added 210 min after termination of mating.

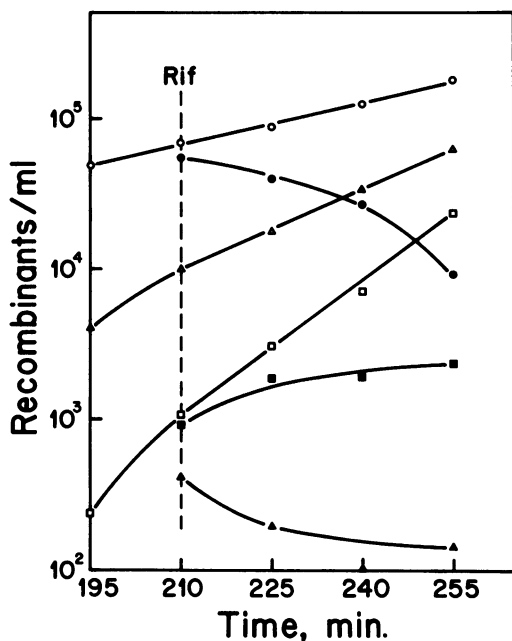


FIG. 7. Effect of rifampin on the phenotypic expression of resistance to colicin E3 and phage BF23 in a sensitive recipient strain (RK4113) after conjugation with a resistant donor strain (RK24). Experimental conditions are the same as Fig. 2. Total *arg*<sup>+</sup> recombinants (○); colicin E3-resistant, *arg*<sup>+</sup> recombinants (Δ); phage BF23-resistant, *arg*<sup>+</sup> recombinants (□); open symbols, a control culture; filled symbols, a culture to which rifampin (100 μg/ml) was added 210 min after the termination of mating.

nants (Fig. 7). As was observed for both spectinomycin and chloramphenicol, the number of phage BF23-resistant recombinants continued to increase for some time in the presence of rifampin. The effect of rifampin on the number of phenotypically colicin-resistant recombinants was quite surprising. Immediately upon the addition of the antibiotic, the number of colicin-resistant recombinants dropped to a level less than 10% the number of phage-resistant ones or, in other words, a complete reversal of the situation as it existed immediately prior to the addition of the antibiotic. (Zero time in the figure actually represents a 5-min incubation with colicin E3 and rifampin [see Materials and Methods].) Thus, though the effect of rifampin was similar to that of chloramphenicol in that they both initiated a decline in the total number of colicin-resistant recombinants, the effect seen with rifampin was much more rapid and, over a 45-min period, occurred to a significantly greater degree.

Sensitivity to colicin E3 in the presence of

chloramphenicol or rifampin is receptor mediated. The effect of chloramphenicol or rifampin on colicin sensitivity observed in the above experiments could be explained by a nonspecific penetration of the colicin E3 molecule through the outer membrane to some lethal site on the cytoplasmic membrane. Inhibition of protein synthesis has been reported to increase the permeability of the outer membrane (19, 26), though this would not explain why the effect was not observed with spectinomycin or why the effect with rifampin was more extensive than that of chloramphenicol.

Several *arg*<sup>+</sup> recombinants from the mating of strain RK24 with strain RK4113 were cloned several times and shown to be resistant to both colicin E3 and phage BF23. Attempts to demonstrate sensitivity of those clones to colicin E3 after the addition of chloramphenicol or rifampin were unsuccessful. This suggested that the effect of these antibiotics being observed on the recombinant population after mating was not nonspecific but rather resulted from the specific interaction of colicin E3 with its receptor on the cell surface. If this were, in fact, the case, then the effect of rifampin is particularly interesting, since this demonstrates that a cell can be resistant to phage BF23 yet still not be devoid of specific receptor molecules on its cell surface, as shown by the sensitivity of these cells to colicin E3.

As this is a fairly important point, the following experiments were done to verify the observation concerning rifampin. (i) Since resistant clones did not become sensitive to colicin E3 after rifampin treatment, there could be some unique feature of colicin-resistant exconjugant cells which makes these cells non-specifically sensitive to the colicin in the presence of certain antibiotics for some time after mating. To test this hypothesis, a resistant male (RK24) was mated with a resistant female (RK4116) in an experiment which was otherwise identical to the one described for Fig. 7. No sensitivity to colicin E3 was observed in the recombinant population after the addition of rifampin 210 min after mating.

(ii) The return to a colicin-sensitive state resulting from rifampin treatment (Fig. 7) might be due to the presence of intact receptor molecules on sensitive recombinants or unmated recipient cells in the population, or receptor molecules these cells may have released into the culture medium. If, in the above experiment, a second female strain that was *argH bfe*<sup>+</sup> but which could be distinguished from the *bfe* female strain by different auxotrophic requirements was present during the mating, there

was still no effect of rifampin on the colicin resistance of *arg*<sup>+</sup> recombinants of the *bfe* female strain.

(iii) It was shown that if the donor strain were rifampin resistant (*rpoB* is closely linked to *argH* and *bfe*), then rifampin had no effect on the sensitivity of phenotypically colicin E3-resistant recombinants to colicin E3. Thus, the effect observed with rifampicin in *rpoB*<sup>+</sup> recombinants must be a direct result of the inhibition of ribonucleic acid synthesis by the antibiotic.

(iv) If the sensitivity resulting from rifampin treatment is indeed receptor mediated, then the recombinant cells should eventually remain resistant to the colicin despite the presence of the antibiotic, due to dilution of the receptors during logarithmic outgrowth of the recombinants. In addition, sensitivity which is receptor mediated should still be inhibited by vitamin B<sub>12</sub>. It was shown that rifampin added to the recombinant population at various times after mating had a differential effect on the colicin resistance of the recombinants (Table 2). When rifampin was added 270 min after mating, greater than 95% of the cells were sensitive to colicin E3. As late as 360 min after mating, when nearly 90%

TABLE 2. Expression of colicin E3 resistance by *arg*<sup>+</sup> recombinants treated with rifampin at various times after mating

Sample and treatment:	Recombinant cells expressing resistance			
	210 <sup>a</sup>	270	360	450
Controls:				
% of total recombinants that are E3 resistant	12.2 <sup>b</sup>	25.9	88.0	100
% of E3-sensitive cells that are protected against E3 by 5 $\mu$ M B <sub>12</sub>	61.9	70.0	97.0	100
Samples tested immediately after addition of rifampin <sup>c</sup> to the recombinant culture:				
% of total recombinants that are E3 resistant	1.2	9.3	82.7	100
% of E3-sensitive cells that are protected against E3 by 5 $\mu$ M B <sub>12</sub>	62.0	70.0	95.0	100
Samples tested 45 min after addition of rifampin to the recombinant culture:				
% of total recombinants that are E3 resistant	<1.0	4.9	39.5	100
% of E3-sensitive cells that are protected against E3 by 5 $\mu$ M B <sub>12</sub>	34.0	39.4	62.9	100

<sup>a</sup> Minutes after mating.

<sup>b</sup> All calculations are corrected for the fact that only 93% of the *arg*<sup>+</sup> recombinants actually became *bfe*.

<sup>c</sup> Rifampin was used at a concentration of 100  $\mu$ g/ml.

of the cells in the control culture were colicin resistant, some 60% of the recombinants expressed sensitivity to the colicin after rifampin addition. However, 100% of the *bfe* recombinant population eventually became resistant to colicin E3 despite the presence of rifampin. Additionally, B<sub>12</sub> did still specifically protect against the lethal adsorption of the colicin when rifampin was present.

**Experiments with colicin E2.** Since colicin E3 is an inhibitor of protein synthesis (24) as are spectinomycin, chloramphenicol, and rifampin, it was of interest to determine if similar effects would be observed with these antibiotics on the sensitivity of recombinants to a colicin with a different mode of killing. Colicin E2 has been shown to degrade deoxyribonucleic acid and inhibit cell division in *E. coli* (24). In experiments similar to those described above, the results observed with colicin E2 were exactly identical to those previously observed with colicin E3.

## DISCUSSION

The evidence presented in this paper strongly suggests that the protein coded for by the *bfe*<sup>+</sup> locus of *E. coli* K-12 can exist in several different states in the outer membrane. These different states are detected by specifically turning "on" or "off" the synthesis of the *bfe*<sup>+</sup> gene product as a result of molecular events after conjugal entry into the cell of a wild-type or mutant allele. One of these states was demonstrated by the introduction of the *bfe*<sup>+</sup> allele into a *bfe* recipient (Fig. 1). Shortly after the transfer of genetic information, 100% of the *arg*<sup>+</sup> *bfe*<sup>+</sup> recombinants became sensitive to both colicin E3 and phage BF23. This result is taken to mean that newly made receptors, when incorporated into the outer membrane, are fully capable of lethally adsorbing either the colicin or the phage.

A second state was demonstrated by the reverse experiment, that is, the introduction of a *bfe* allele into a *bfe*<sup>+</sup> recipient (Fig. 2). Colicin E3-resistant recombinants were observed significantly earlier than phage BF23-resistant recombinants, despite the fact that both of these lethal agents utilize the same receptor. The detection of phenotypically colicin-resistant phage-sensitive recombinants did not result from a failure of these cells to adsorb the colicin (Table 1); thus it would appear that the *bfe*<sup>+</sup> gene product can naturally exist in a colicin-tolerant state on the surface of an otherwise Tol<sup>+</sup> cell.

It would appear that the transition from a colicin-sensitive state to a colicin-tolerant state



requires protein synthesis, since the inhibition of protein synthesis by the antibiotic spectinomycin prevented any further increase in the number of phenotypically colicin-resistant cells in the recombinant population (Fig. 5), although the recombinants continued to divide for some time in the presence of the antibiotic. It was interesting that, after the addition of spectinomycin, the number of phage-resistant cells continued to increase until it just about equaled the number of colicin-resistant cells, suggesting that the colicin-tolerant state was a precursor to a phage-resistant state, a transition which was apparently not dependent on protein synthesis. That the receptor can actually exist in such a "resistant" state, that is, in a condition where it is incapable of lethally adsorbing either colicin E3 or phage BF23 but is still present, presumably in the outer membrane, is supported by two additional experiments. (i) Since the number of recombinants resistant to colicin E3 or phage BF23 continued to increase after the inhibition of cell division by nalidixic acid (Fig. 4), it would appear that resistance is not wholly due to the distribution of receptor molecules to daughter cells during cell division in such a way as to generate cells devoid of receptor activity. Resistant recombinants could continue to arise under these conditions as a result of protein turnover in the outer membrane. In this regard, protease activities have been identified in the envelope fraction of *E. coli* (25). A second possibility is that the functional receptors remaining on the surface of these cells are somehow altered so that they are no longer capable of lethally adsorbing either colicin or phage. (ii) There is a finite period of time after mating in which recombinant cells which have phenotypically expressed resistance to both colicin E3 and phage BF23 can be specifically made sensitive to colicin E3 after the addition of the antibiotic rifampin (Fig. 7). This period of time is roughly equivalent to that period in which the recombinants would, by statistical distribution, still retain receptors on their cell surface.

To explain these results, one can envision several different "sites" for the receptor on the cell surface. The newly synthesized receptor emerges at a site where it is capable of lethally adsorbing both colicin E3 and phage BF23. After an undetermined period of time and as a result of some process requiring protein synthesis, the receptor enters a "transition" state where, for a time, it is capable of lethally adsorbing only the phage, though still able to bind the colicin. Eventually this transition results in the receptor being unable to lethally adsorb

either the colicin or the phage. Its continued presence on the cell surface, however, can be demonstrated by the addition of rifampin which, by inhibiting ribonucleic acid synthesis, results in a perturbation of the cell surface that now permits the receptor to specifically and lethally adsorb colicin E3.

The basis for the differential effects observed after the addition of the antibiotics spectinomycin, chloramphenicol, and rifampin to the recombinant population at various times after mating is not understood and is being further investigated. There would not appear to be any correlation with the results of Hirashima et al. (13), who compared the inhibitory effects of various antibiotics on envelope and cytoplasmic protein synthesis. Although other workers have observed that protein synthesis inhibition can lead to alterations in the surface properties of *E. coli*, such as sensitization to actinomycin D (26), we are quite convinced that the sensitization to colicin E3 observed in recombinant cells resulting from rifampin treatment is receptor mediated. Dworsky and Schaechter (8) have shown that rifampin treatment markedly decreased the number of deoxyribonucleic acid-membrane attachment sites in *E. coli*, so it is conceivable that the loss of these attachment sites somehow affects protein arrangement in the outer membrane. Despite the lack of a firm biological basis, the effects observed with these antibiotics would suggest that the outer membrane of *E. coli* is not a particularly static structure.

The demonstration of several different states for the colicin E receptor on the cell surface provides a physiological basis to explain the results of Shannon and Hedges (29), who showed that there are both lethal and nonlethal sites on the surface of wild-type *E. coli* for the specific adsorption of colicin E2. It might also explain why certain bacteriophages irreversibly adsorb to *E. coli* only in the region of so-called "adhesion sites" between the cytoplasmic and outer membranes, although these sites represent only about 5% of the total cell surface (1, 2). It has been suggested that proteins are specifically inserted into the outer membrane at these adhesion sites (22), and it is tempting to speculate that the interplay of the *bfe*<sup>+</sup> gene product with these sites could explain the transitions described in this paper.

Leal and Marcovich (20) reported the results of an experiment in which they introduced resistance to phage T6 into a sensitive recipient by conjugation, scoring for *tsx pro*<sup>+</sup> recombinants. Their results were very similar to those described herein for phage BF23 resistance.

They interpreted their results to mean that the appearance of phage T6-resistant recombinants beginning shortly after the onset of cell division after mating was due to a nonrandom segregation of receptor molecules to daughter cells. This was in contrast to other experimental approaches which suggested that membrane growth in *E. coli* was of a random mode (18). Phage T6 and colicin K utilize the same receptor (5). Had Leal and Marcovich scored for both colicin K- and phage T6-resistant recombinants, they might have observed results similar to those reported in this paper for colicin E3 and phage BF23, results requiring a different interpretation.

In conclusion, evidence that the receptor for the E colicins and phage BF23 can exist in different functional states in the outer membrane of *Escherichia coli* has been presented. Due to the nature of these experiments, it was impossible to determine how these results relate to the primary biological role of the *bfe*<sup>+</sup> product, that being the transport of vitamin B<sub>12</sub>. Could it be, for example, that only newly synthesized receptors are involved in the active transport of B<sub>12</sub>? An attempt is currently under way to answer that question from a somewhat different experimental approach.

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