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Competition Between Congenic *Escherichia coli* K-12 Strains In Vivo

A. ONDERDONK,[1,2]* B. MARSHALL,[2] R. CISNEROS,[1] AND S. B. LEVY[2]

School of Veterinary Medicine[1] and School of Medicine,[2] Tufts University, Boston, Massachusetts 02111

The ability of *Escherichia coli* to colonize the large bowels of animals is related to many factors inherent to the intestinal environment and the bacterium. The use of germfree mice eliminates the competition between *E. coli* and the other microflora and allows most *E. coli* strains to colonize. We found that *E. coli* K-12 strains differing in chromosomal antibiotic resistance could monoassociate in germfree mice in large numbers. However, when two or more strains were in competition with each other, we detected quantitative differences in the abilities of the strains to colonize. The order of colonizing ability was as follows: nalidixic acid resistance > streptomycin resistance > rifampin resistance. We also found that a nalidixic acid-resistant strain bearing plasmid pBR322 colonized less efficiently and at lower levels when in competition with the nalidixic acid-resistant strain. Studies of the membrane proteins of the various strains indicated that changes in membrane proteins occurred concomitantly with altered resistance to antimicrobial agents. These results suggest that chromosomally linked alterations in antimicrobial sensitivity may also reflect changes in membrane proteins and a decreased ability to colonize mammalian intestines in otherwise isogenic bacterial strains.

The intestinal microflora represents a unique ecological system composed of hundreds of different species of bacteria (4). The interrelationships among these species and the overall impact of this microflora on a host are often essential to the normal functioning of the host. Of particular interest in defining this microflora is an understanding of the factors which influence the colonization of the host with new species or strains of bacteria. Since many infections result from contamination of host tissues with members of their own normal microflora, the characteristics of the species which make up this microflora are of considerable interest.

In recent years, detailed genetic characterizations of certain species, such as *Escherichia coli*, have provided researchers with the tools necessary to address some of these questions. Strains of this species that are genetically identical except for chromosomally linked antibiotic resistance have been derived in a number of laboratories. This report describes a study in which these strains were compared in vivo for their relative abilities to colonize mice. Germfree mice were used as the in vivo system in order to avoid possible colonization resistance factors, which may interfere with establishment when an intact microflora is present (1).

MATERIALS AND METHODS

Animals. Male, germfree CD-1 mice weighing between 14 and 18 g were used for all experiments (Charles River Breeding Laboratories, Wilmington, Mass.). Animals were maintained in plastic isolators and were given a diet of chow (Agway) and water ad libitum. The bacteriological sterility of each group of mice was checked by swabbing the fur, feet, anus, mouth, and ears of representative animals and by obtaining fecal pellets from randomly selected animals. Swabs and fecal pellets were placed into chopped meat glucose broth (Scott Laboratories, Fiskeville, R.I.) and incubated at 37°C in an anaerobic chamber (Labline Instruments, Melrose Park, Ill.) for 48 h. Subcultures of each tube were then plated onto Brucella base blood agar and MacConkey agar and incubated for an additional 48 h. The absence of growth on any of the plates or in the media in tubes was interpreted as bacteriological sterility (8).

Bacterial strains. We used a single strain of *E. coli* K-12, strain χ1666 (*ara*$^-$ Nal^r; obtained from R. Curtiss III, University of Alabama, Birmingham), as the wild-type strain in these experiments. Strain χ1666 contains a cryptic plasmid (M. Tachibara and S. B. Levy, manuscript in preparation) which does not code for detectable antibiotic resistance or colicin production. A rifampin-resistant mutant of this strain, χ1666 Nal^r Rif^r, and a streptomycin-resistant mutant, χ1666 Nal^r Sm^r, were selected in vitro by stepwise plating onto media containing increasing concentrations of the respective drugs. D20-5, a pBR322 derivative of χ1666, was also used (6). Before use, frozen stock cultures of each strain were thawed, inoculated into 5 ml of brain heart infusion broth, and incubated for 4 to 6 h at 35°C in a shaking water bath. Cultures were checked for purity by using appropriate selective media before they were placed into the plastic isolators for association with germfree mice. We detected no

additional plasmids in the derivative strains, except pBR322 and D20-5.

Association of mice with bacterial strains. Groups of mice were given the various bacterial strains by orogastric administration of 0.1 ml of a culture containing 10^9 colony-forming units (CFU) per ml. After this association, the bacterial population density was monitored by obtaining fecal pellets from four randomly selected animals, pooling the material in sterile saline, and performing quantitative bacteriological cultures. Previous studies with χ1666 and χ1666 Rif^r in which 10 animals were monitored daily during mono- and di-associations revealed that the largest standard deviations of the mean were $10^{0.38}$ for χ1666 and $10^{0.72}$ for χ1666 Rif^r. A *t* test of dependent means between χ1666 and χ1666 Rif^r during di-associations indicated that a significant difference ($P < 0.05$) existed between the two means. We also found that pooled sample counts obtained at the same time yielded counts virtually identical to the means obtained with individual samples.

Bacteriology. Quantitative bacteriological assessments of the various strains present in pooled fecal material were performed by making serial 10-fold dilutions of the pooled feces in sterile phosphate-buffered saline (pH 7.2) after the sample was weighed. A 0.1-ml portion of each dilution was plated onto MacConkey agar containing the appropriate antibiotic. For χ1666, MacConkey agar containing 30 μg of nalidixic acid per ml was used; for χ1666 Rif^r, colonies from MacConkey agar plates were replicated onto agar containing 30 μg of nalidixic acid per ml and 10 μg of rifampin per ml, and for χ1666 Sm^r, colonies were replicated onto agar containing 30 μg of nalidixic acid per ml and 100 μg of streptomycin per ml. Additional MacConkey agar plates containing no antibiotics were also used to compare the total viable cell counts with the cell counts obtained when media containing antibiotics were used. Plates were incubated at 37°C for 48 h, and colonies were subcultured and identified as a check of culture purity. All cell counts were expressed as colony-forming units per gram of fecal material.

Sodium dodecyl sulfate-polyacrylamide gel analysis and agarose gel studies. Bacterial cultures were labeled with [^{35}S]methionine or ^{14}C-amino acids as described previously (3). Membranes were prepared by ethylenediaminetetraacetate-lysozyme treatment (3–7) and then solubilized in sodium dodecyl sulfate buffer (3). Gel analyses were performed as described previously, and autoradiography was done by using Kodak film (3). The presence or absence of plasmids was determined by using lysed cells in 1% agarose gels (Y. Machida and E. Ohtsubo, personal communication).

Experimental design. Five groups of experimental animals were associated with χ1666 and one or more of the chromosomally mutant resistant strains derived from χ1666. The first group was associated with χ1666 and subsequently with χ1666 Sm^r. The second group received the same two strains in reverse order, followed by a wild-type mouse *E. coli*. The third group received χ1666 first and subsequently χ1666 Rif^r. The fourth group of animals received χ1666 Rif^r, then χ1666 Sm^r, and finally D20-5. The fifth group received the same two initial strains in reverse order, followed by D20-5.

RESULTS

Association of mice with χ1666 and χ1666 Sm^r. After orogastric association of mice with χ1666, the population density in the large intestine reached 3×10^7 CFU/g within 24 h. By day 8, this population had stabilized at 2×10^9 CFU/g, and it remained at this level for the duration of the experiment (Fig. 1). On the other hand, association of these animals with χ1666 Sm^r on day 8 resulted in the establishment of this strain at a viable cell density of only 9×10^5 CFU/g by 24 h after association. A slow but steady decline in this population occurred over the next 10 days to a mean concentration of 10^3 to 10^4 CFU/g. These data indicate that these two strains, which differed phenotypically only in the expression of a chromosomal mutation for drug resistance, colonized a gnotobiotic host with different proficiencies when both were present in vivo simultaneously.

To determine whether the inability of χ1666 Sm^r to colonize gnotobiotic mice was simply a reflection of competition for nutrients between these two similar strains, the order of association for the two strains was reversed. A group of mice were associated first with χ1666 Sm^r and then, on day 7, with χ1666. As with χ1666 alone, the Sm^r strain colonized mice well (6×10^9 CFU/g) within 48 h after association (Fig. 2). Association of these animals with χ1666 on day 7 resulted in stable populations of both strains at viable cell densities of 10^9 CFU/g by day 10. Of particular interest in this experiment was the establishment of χ1666 at a viable cell density comparable to that of χ1666 Sm^r. Both populations remained at comparable levels for the duration of the experiment. As an additional check on the sta-

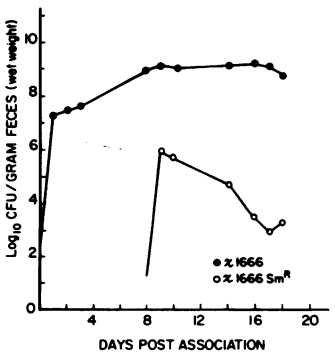

FIG. 1. *Colonization of germfree mice by χ1666, followed by colonization by χ1666 Sm^r. Collection of mouse feces and assays were performed as described in the text.*

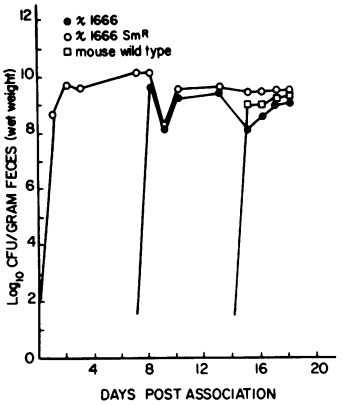

FIG. 2. *Colonization of germfree mice by χ1666 Sm^r, followed by colonization by χ1666 and a wild-type mouse E. coli. Methods were as described in the text.*

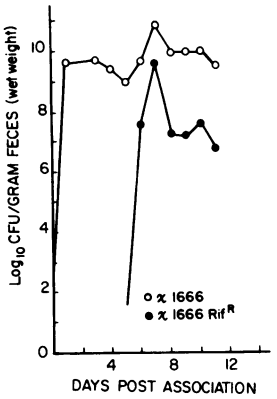

FIG. 3. *Colonization of germfree mice by χ1666, followed by colonization by χ1666 Rif^r. Methods were as described in the text.*

bility of this association, these animals were challenged with a wild-type, naturally occurring *E. coli* strain obtained from mice on day 14. This wild-type strain established itself at a level comparable to the levels of both χ1666 and χ1666 Sm^r. All three strains remained at essentially the same viable cell density for the ensuing 4 days, after which the experiment was terminated. The wild-type *E. coli* strain did not dislodge the two already established *E. coli* strains, nor did it result in a decreased viable cell density for either of these two strains.

Association of mice with χ1666 and χ1666 Rif^r. To determine whether the inability of χ1666 Sm^r to colonize in large numbers when χ1666 was already present was due to a unique feature of the Sm^r marker, we tested a second derivative of χ1666. A group of mice was associated first with χ1666 and then with χ1666 Rif^r. Whereas χ1666 again colonized at $>10^9$ CFU/g, χ1666 Rif^r colonized at a stable level which was considerably lower (10^6 to 10^7 CFU/g) (Fig. 3). These data indicate that both χ1666 mutants with chromosomal antibiotic resistance markers had diminished abilities to colonize mice when χ1666 was already present.

Association of mice with χ1666 Rif^r and χ1666 Sm^r. Subsequent experiments were designed to determine whether the inability of χ1666 chromosomal mutants to colonize occurred only when these mutants were in competition with the parent strain. The χ1666 Rif^r and χ1666 Sm^r strains were compared in vivo for their relative abilities to colonize in the presence of a previously established population of the other strain. Two groups of mice were associated sequentially with χ1666 Rif^r and χ1666 Sm^r. The first strain used colonized in numbers exceeding 10^9 CFU/g, whereas the second strain colonized in lower numbers (Fig. 4 and 5). χ1666 Sm^r achieved a maximum viable cell density of 10^4 to 10^5 CFU/g when it competed with χ1666 Rif^r, and χ1666 Rif^r was detected only sporadically at viable cell densities of more than 10^2 CFU/g (the lowest detectable limit) when it was in competition with χ1666 Sm^r. These data indicate that the colonization proficiencies of χ1666 Sm^r and χ1666 Rif^r in mice are related to the order of association. A similar colonization pattern was not observed when the parent strain (χ1666) was used. With strain χ1666, no apparent effect on colonization was found in the presence of isogenic strains (Fig. 2).

To determine whether *E. coli* strains carrying plasmid-mediated antibiotic resistance affected colonization, mice were also associated with D20-5, a derivative of χ1666 bearing the nonconjugative plasmid pBR322 (bearing resistance to tetracyline and ampicillin). In this case, D20-5 was unable to colonize in large numbers when another strain was already present (Fig. 4 and 5). When mice were associated with χ1666 Rif^r and χ1666 Sm^r and then D20-5, the plasmid-bearing strain reached viable cell densities of only 10^7 CFU/g (Fig. 4). When χ1666 Sm^r and χ1666 Rif^r were used, D20-5 failed to colonize at viable cell densities of more than 10^2 CFU/g. However, if D20-5 alone was used, the levels of colonization were 10^9 to 10^{10} CFU/g (6; data not shown).

Membrane differences among *E. coli* derivatives. Since cell surface differences could affect the abilities of the organisms to remain associated with murine intestines, different cell fractions of strain χ1666 and its chromosomally resistant derivatives were examined for any differences. The three strains were labeled overnight in medium containing [^{35}S]methionine;

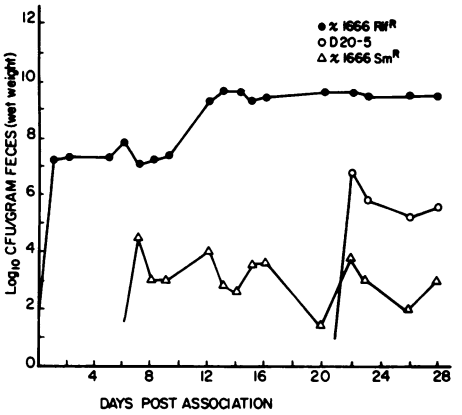

FIG. 4. *Colonization of germfree mice by χ1666 Rif^r, followed by colonization by χ1666 Sm^r and then D20-5. Methods were as described in the text.*

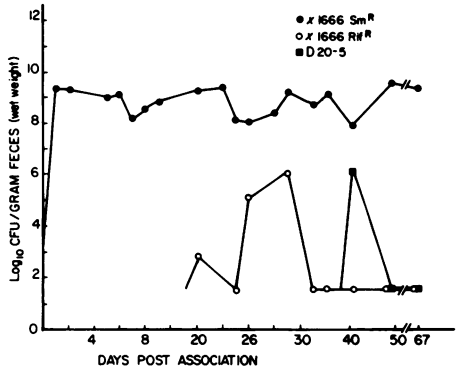

FIG. 5. *Colonization of germfree mice by χ1666 Sm^r, followed by colonization by χ1666 Rif^r and then D20-5. Methods were as described in the text.*

then the cells were washed and lysed, and the resulting material was separated into an inner membrane fraction (Sarkosyl extracted), an outer membrane fraction, and a supernatant fraction. We detected no differences in the supernatant proteins (data not shown). No obvious changes were observed in the major cellular outer membrane proteins (e.g., porins, II*, and lipoprotein). However, changes in other proteins were detected (Table 1 and Fig. 6) when the mutants were compared with χ1666. The rifampin-resistant strain showed at least two changes: an increase in outer membrane protein c and the loss of a 70,000-dalton outer membrane protein (protein d). This latter protein was also absent from χ1666 Sm^r. The streptomycin-resistant strain showed other changes. Four proteins increased in amount; three of these were located preferentially in the outer membrane. One new protein appeared (protein f, a 30,000-dalton inner membrane protein), and a 20,000-dalton outer membrane protein (protein h) was missing. These data indicate that cell membrane protein changes occurred in conjunction with altered antimicrobial resistance in the strains derived from χ1666.

DISCUSSION

Our results indicate that strain-specific factors are responsible for the ability of strains derived from χ1666 to colonize germfree murine large intestines. We have shown that the parent strain, strain χ1666, is capable of colonizing at levels equal to the levels of wild-type mouse *E. coli* strains and that this strain is not affected by the presence of other strains derived from χ1666. On the other hand, both χ1666 Sm^r and χ1666 Rif^r colonize at lower population densities in the presence of an already established χ1666 population compared with colonization by each strain alone. This effect does not appear to be due to competition for nutrients, since both χ1666 Sm^r and χ1666 Rif^r colonize in large numbers in the presence of χ1666 if their colonization precedes exposure to χ1666 (Fig. 2). In addition, D20-5, a χ1666 derivative bearing a nonconjugative plasmid, is not as successful in colonizing mice previously colonized with other χ1666 strains, although D20-5 alone colonizes in large numbers.

Previous studies with mice given streptomycin to eliminate sensitive bacteria have shown that *E. coli* K-12 does not colonize animals as well as human fecal *E. coli* strains (2). It has also been shown that *E. coli* J5-3, an F^- strain of *E. coli* K-12, colonizes these animals almost as well as the human *E. coli* strain. These data clearly suggest that the various strains of *E. coli* used have different abilities to colonize a "sensitive" host. Since suppression of the enteric microflora with streptomycin does not necessarily alter the ability of obligate anaerobes to contribute to colonization resistance, little information regarding intrastrain colonization resistance factors can be gained from these previous studies, although the data suggest that colonization is related to the presence of other *E. coli* strains (2). However, such intraspecies colonization resistance factors have been shown to be present for other species, such as *Staphylococcus aureus* (9). Orcutt and Schaedler (9) showed that colonization of mice with one strain of *S. aureus*, strain 502A, resulted in a decreased ability for a second more virulent strain to coexist. In this instance, the inability of the second strain to colonize was not caused by just a competition for nutrients or colonization sites, since both strains colonized equally if the order of association was reversed.

Although it would be appealing to postulate that the host in our experiments played some

TABLE 1. *Differences in membrane proteins in congenic E. coli strains*

Membrane protein[a]	Approx mol wt ($\times 10^3$)[a]	Location[b]	Strains[c]	
			χ1666 Rif^r	χ1666 Sm^r
a	110	O > I	ND	IP
b	100	O	ND	IP
c	77	O	IP	ND
d	70	O	−c	−
e	59	O	ND	IP
f	30	I	ND	+
g	22	I > O	ND	IP
h	20	O	ND	−

[a] Letters refer to the proteins visualized in Fig. 6. Molecular weights were determined by using internal standards (3).

[b] I, Inner membrane; O, outer membrane.

[c] Differences compared with χ1666: IP, increased production; +, new protein; −, protein missing; ND, no detectable difference.

role in colonization resistance, such does not appear to be the case. Numerous studies by a number of investigators have shown that germfree animals can be colonized by a wide variety of bacterial species (10–12). In addition, the theory that one strain somehow blocks "colonization sites" in the large bowel does not seem to apply. We showed that both χ1666 Sm^r and χ1666 Rif^r could coexist in large numbers with χ1666, depending on the order of association. The data presented here suggest that the ability to compete for colonization of the murine gastrointestinal tract is due to changes in a strain which occur in conjunction with an alteration in chromosomally induced antimicrobial resistance. This decreased competition may be related to the membrane protein differences observed among these congenic *E. coli* strains (Fig. 6).

Although chromosomal antibiotic resistance is expressed as a detectable phenotypic marker,

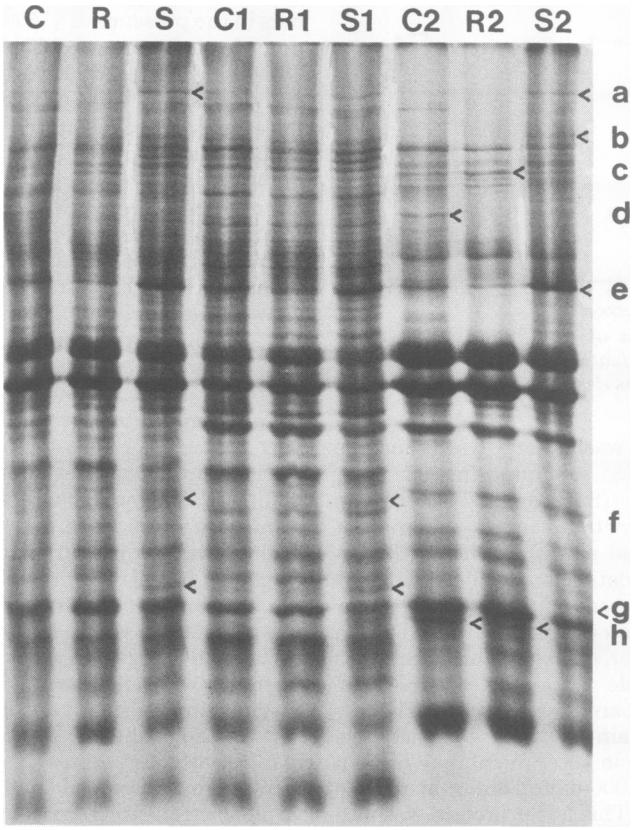

FIG. 6. *Sodium dodecyl sulfate-polyacrylamide gel analysis of ^{35}S-labeled membrane proteins from χ1666 and congenic strains. Lane C, χ1666; lane S, Sm^r derivative; lane R, Rif^r derivative; lane C1, χ1666 Sarkosyl-extracted protein; lane R1, χ1666 Rif^r Sarkosyl-extracted protein; lane S1, χ1666 Sm^r Sarkosyl-extracted protein; lane C2, χ1666 Sarkosyl-insoluble membrane protein; lane R2, χ1666 Rif^r Sarkosyl-insoluble membrane protein; lane S2, χ1666 Sm^r Sarkosyl-insoluble membrane protein. Letters refer to protein bands showing differences (see Table 1).*

it appears to also reflect a more pleiotropic effect. The fact that chromosomally resistant *E. coli* mutants were not able to colonize intestines successfully when in competition with the parent strain suggests that genetic variation may alter colonization proficiency. A further exploration of such strain-specific colonization resistance would be of both clinical and ecological interest.

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