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Survival of *Escherichia coli* Host-Vector Systems in the Mammalian Intestine

Abstract. *Survival in the mouse and human intestine of* Escherichia coli *host-vector systems used and proposed for recombinant DNA technology was assessed. There was no detectable survival of severely disabled* E. coli *K12 strain χ1776 in mice or in human subjects 24 hours after ingestion. The same strain bearing the plasmid pBR322, however, was recovered from human subjects for 4 days in amounts of six organisms for every million ingested. Nondisabled* E. coli *K12 strain χ1666, with or without pBR322, survived in 10^4-fold greater numbers and for 2 days longer, with better recovery of the plasmid-containing derivative. Although the plasmid-bearing strains were recovered for longer periods, no intestinal colonization was noted. Despite the presence of pBR322 for a maximum of 6 days in the human intestine, there was no evidence that it was transferred from either bacterial host to endogenous aerobic fecal bacteria.*

Certain *Escherichia coli* host-vector systems, designated EK2(HV2), have been approved for use in recombinant DNA research. Mutations in both host and vector have been introduced so as to improve biologic containment (*1*-*5*). One particular host cell, χ1776, developed by Curtiss and colleagues (*1*) was derived to be unable to survive outside the defined laboratory environment. It is sensitive to bile acid, resistant to nalidixic acid, and multiply auxotrophic (Dap^-, Thy^-, Met^-, Thr^-, Bio^-). Plasmid vectors, such as pBR322 (*2*), have been constructed that are incapable of directing transfer of themselves to other bacterial hosts and are also poorly transferred by other plasmids.

We have reported the recovery of χ1776 from mice for 2 days after inoculation by different parenteral routes (*6*). The mean lethal dose (LD_{50}) was 10^3- to 10^4-fold greater than that of naturally occurring *E. coli*, but not markedly different from that of a laboratory *E. coli* K12 strain. In the present studies, we compared in mice and in human subjects the survival in the intestinal tract of χ1776 and its plasmid pBR322-bearing derivative, χ2236, with that of a conventional laboratory EK1 strain, *E. coli* K12, χ1666 (Ara^-, Nal^R) (*7*) and its pBR322-bearing derivative, D20-5.

When doses of 5×10^9 χ1776 cells were given by gastric intubation to ten germ-free mice, no intestinal colonization was found. The organism was detected only in fecal samples analyzed within the first 24 hours after inoculation. Similarly no intestinal colonization was found with χ2236 given in food, by gastric intubation, or by rectal administration. In contrast, χ1666 and D20-5 colonized in germ-free mice at 10^9 cells per gram of feces. Inoculations of 5.2×10^9 cells of χ1666 did not lead to colonization of the intestinal tract of conventional mice; cells were recovered only up to 3 days after gastric intubation.

Studies in human subjects were performed on groups of four male volunteers housed in the Clinical Study Unit of Tufts-New England Medical Center Hospital. They were permitted to leave the room only once each day for 1 to 2 hours. No physical contact with other individuals was permitted. An attachment on the toilet ensured that all fecal samples would be collected in an autoclavable plastic bag. These samples were immediately placed in the refrigerator in the room until being taken for analysis.

In the first experiment, three of four individuals drank 1.9×10^9 viable cells of χ1776 in milk on two separate occasions, 6 hours apart. After each dose, skin, nose, and throat swabs were cultured in supplemented eosin-methylene blue broth (EMB) with nalidixic acid (Nal) or on agar plates (EMB-Nal) (*8*) to identify the presence of the ingested organism. Each fecal sample was processed within 1 hour of passage. It was weighed, mixed thoroughly in the plastic bags, and sampled on EMB-Nal agar to determine the number of endogenous and ingested *E. coli* and enterococci per gram of feces.

Up to 1 hour after ingestion, χ1776 was detected in the nose and throat;

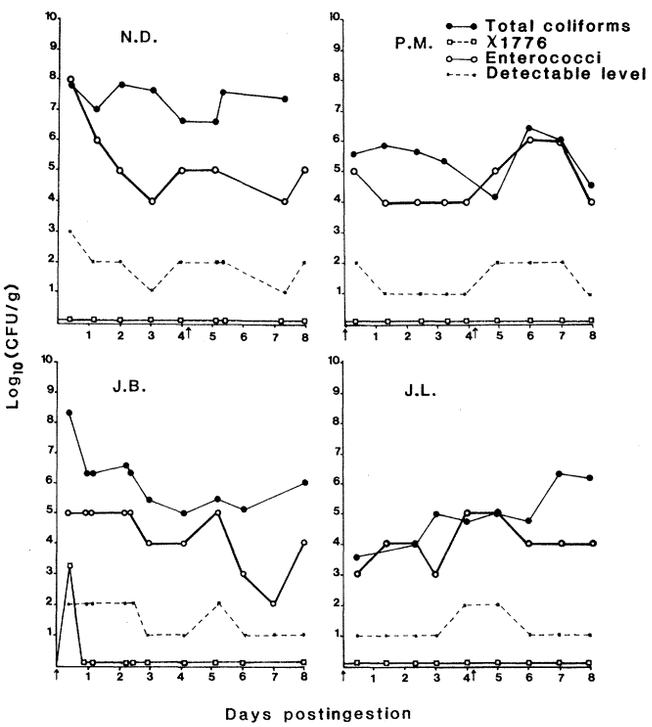

Fig. 1. Survival of χ1776 after the ingestion of 3.8×10^9 organisms on day 0 and 1.5×10^{10} organisms on day 4 (arrow). The minimal detectable level for each sample is designated by the dashed line. The ordinate is number of colony-forming units (*CFU*) of bacteria per gram (wet weight) of feces.

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Table 1. Recovery of ingested organisms in fecal samples. Doses of bacteria ingested: χ2236, 2.3×10^9; D20-5, 9.4×10^9; and χ1666, 8.4×10^9.

Volunteer	Test organism	7-day fecal sample (g)		Total excretion of test organisms (No.)	Recovery rate
		Total	Amount containing any test organism		
R.D.	χ2236	820	200	3.0×10^4	1.3×10^{-6}
J.A.	χ2236	1015	528	9.5×10^4	4.1×10^{-6}
C.L.	χ2236	1418	914	2.1×10^5	9.1×10^{-6}
L.B.	χ2236	1768	957	2.1×10^5	9.1×10^{-6}
Mean	χ2236	1255 ± 423	650 ± 357	$1.4 \pm 0.89 \times 10^5$	$5.9 \pm 3.87 \times 10^{-6}$
M.W.	D20-5	807.5	869	8.2×10^7	8.7×10^{-3}
	χ1666	941	556	1.4×10^6	1.6×10^{-4}
R.C.	D20-5*	1167	1168	9.4×10^8	2.0×10^{-1}
	χ1666 (not fed)	1022			
D.O.	D20-5	833	606	1.2×10^8	1.3×10^{-2}
	χ1666	1166	516	2.0×10^7	2.4×10^{-3}
A.J.	D20-5	1421	1421	1.2×10^8	1.3×10^{-2}
	χ1666	1498	1254	1.6×10^8	2.0×10^{-2}
Mean for D20-5		1057 ± 293	1016 ± 354	$3.2 \pm 4.2 \times 10^8$	$5.9 \pm 9.4 \times 10^{-2}$†
Mean for χ1666		1157 ± 246	775 ± 415	$6.2 \pm 9.0 \times 10^7$	$0.75 \pm 1.1 \times 10^{-2}$

*Only 4.7×10^9 organisms were ingested. †Mean recovery rate excluding R.C. = $1.2 \pm 0.25 \times 10^{-2}$.

none was ever isolated from the hands. Recovery of χ1776 in feces appeared to be affected by the presence of $> 10^5$ enterococci on the plate. In separate studies (see below), we determined the minimum detectable amount of χ1776 for each fecal sample (Fig. 1). The organism was detected only once in the feces from one individual 5 hours after ingestion (Fig. 1). In a second ingestion, on day 4, three of the volunteers drank ten times more χ1776 (1.5×10^{10}). No χ1776 was detected in the fecal samples (Fig. 1). No spread of the bacterium was found from the three ingestors to the one noningestor.

In a second experiment, four different volunteers swallowed χ2236. Doses of 1.6×10^{10} cells and 7×10^9 cells were ingested 6 hours apart. No organisms were detected on the skin, but organisms were recovered from the nose in one instance 15 minutes after ingestion. For 2 to 5 hours, organisms could be recovered from the throat. In marked contrast with χ1776, χ2236 was detected in feces for 3.5 to 4 days from all volunteers in amounts ranging from 80 to 400 bacteria per gram of feces (Fig. 2). These bacteria constituted about 0.01 percent of the total *E. coli* present in the feces. Six bacteria of every million ingested were recovered (Table 1). Of approximately 200 presumptive χ2236 colonies recovered, 125 were subcultured; all, like χ2236, were minicell formers, and all showed the same auxotrophic requirements of the original χ2236. Representative isolates from each volunteer were examined to confirm the identity of each isolate with the host χ1776; only one plasmid, pBR322, was present in these recovered organisms (*9*).

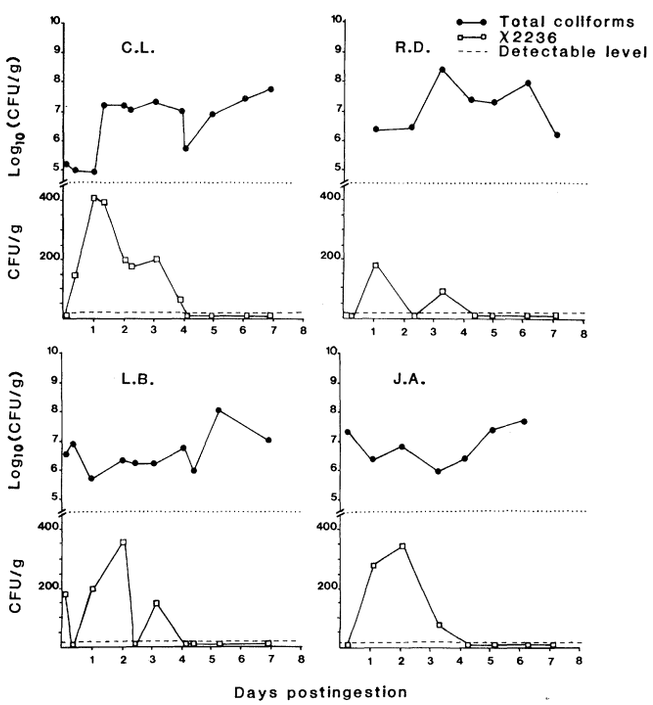

Fig. 2. Survival of χ2236 after the ingestion of 2.3×10^{10} organisms. The minimal detectable level was 10 CFU per gram (wet weight) of feces.

There are difficulties in comparing the survival of the two strains due to the differences in retrieval systems and the dosages given. The amounts of feces evaluated, however, was the same. In both groups, daily excretions were 162 ± 19 g. The χ2236 was recoverable on media containing tetracycline and ampicillin at a minimal detection level of ten organisms per gram of feces. The recovery of χ1776 on EMB-Nal plates was affected, however, by Nal^R enterococci that could not be eliminated from the selection plate.

In order to assess the effect of enterococci on χ1776 recovery, we performed reconstitution experiments with fecal samples from five different individuals. The results, as summarized in Table 2, indicated that with fewer than 10^5 enterococci per gram, recovery would be similar to that with χ2236, namely, ten organisms per gram of feces. With enterococci numbering between 2×10^5 and 6×10^7, recovery would be possible only if there were ≥ 100 organisms per gram. Ingestors of χ1776 received smaller doses of cells (16.5 and 65.2 percent, respectively, of the χ2236 dose). Survivors from the first dose would not have been detectable. Organisms should have been detected after the second dose if survival were similar to that with χ2236. In five of the 12 samples, the minimum detectable amount was ten organisms per gram; in the other seven, it would have been 100 to 200 per

gram. Furthermore, the highest concentration of organisms per dose (the first dose of χ2236 and the single dose of χ1776) were the same. Consequently we might have expected similar survivals during the first 24 to 48 hours after ingestion. This was not the case. These results suggest therefore that the plasmid had enhanced the survival of χ1776.

We then studied survival of *E. coli* K12 D20-5 and χ1666. Four volunteers ingested two doses of D20-5 6 hours apart (4.7×10^9 cells per dose). Recovery in the mouth lasted 1 to 3 hours; little was recovered from the nose, and none from the skin. Compared with χ2236, D20-5 survived at a rate 10^4 times higher and for about 2 days longer (Table 1 and Fig. 3). To determine whether the plasmid had affected survival, we then fed the host bacterium χ1666 (8.4×10^9) to the same volunteers. The overall survival rate of χ1666 was significantly less than that of D20-5 in two volunteers, but the same in one (J.A.) (Table 1 and Fig. 3).

In no instance was intestinal colonization found. We used two approaches to determine whether plasmid pBR322 had been transferred to endogenous bacterial aerobes during transit through the human intestinal tract. First, we evaluated whether pBR322 had in fact become transferable in any of the isolates by virtue of the host organism having picked up a mobilizing genetic element. When 116 of the recovered χ2236 organisms and 4000 of the recovered D20-5 organisms were plated with laboratory *E. coli* recipient, χ984, on nonselective plates and then replica-plated to selective media that would allow only the recombinant to grow, none of the isolates was able to transfer the plasmid.

Second, we used DNA-DNA hybridization of a ^{32}P-labeled restriction enzyme fragment of the tetracycline resistance determinant on pBR322 (*10*) to determine the presence of the plasmid in isolated bacterial colonies. Studies of individuals in the Boston area have shown that this determinant is relatively uncommon (< 10 percent) (*11*). Its combination with Amp^R was sufficiently unusual to make these two markers useful in identifying initially the presence of pBR322. We quantitatively and qualitatively selected and isolated those fecal organisms bearing both tetracycline and ampicillin resistance: a gram of feces was inoculated into 100 ml of Todd-Hewitt broth, containing tetracycline (10 μg/ml) and ampicillin (50 μg/ml), or it was diluted serially and plated on blood agar and phenylethyl alcohol plates, with the same drugs added. We collected 81 isolates of representative endogenous coliforms from the eight volunteers after ingestion: four were Gram-positive and 77 were Gram-negative; there were five different morphology types, including both lactose fermenters and nonfermenters, and at least eight *Pseudomonas* spp. We measured hybridization of the probe to the DNA of the isolates by the colony hybridization method (*12*). None of the 48 colonies from individuals ingesting χ2236 and three of the 33 colonies from individuals ingesting D20-5 showed some degree of hybridization. One of the latter closely resembled D20-5 in being Ara$^-$, Nal^R and having identical biochemical tests in API20E strips (Analytab Products). It contained plasmid pBR322 (by agarose gel analysis), but was mucoid. This morphology was lost at 40°C as has been described for mucoid *E. coli* variants (*13*). This evidence, in addition to the unusual coexistence of the Ara$^-$ and Nal^R characteristics indicated that it was a mucoid variant of D20-5. The other two colonies showed only faint evidence of hybridization and neither contained plasmid pBR322; by gel analysis, plasmids of sizes different from pBR322 were seen.

Table 2. Varying numbers of χ1776 were added to a gram of feces suspended in 9 ml of sterile buffered saline, and serial dilutions were plated on EMB-Nal. The enterococci titer was determined, and the number of χ1776 colonies recovered was compared with that obtained from similar dilution platings of χ1776 in saline alone.

Test	Enterococci (number per gram)	Colonies of χ1776 required for minimal detection (number per gram)
1	5×10^4	10–13
2	2×10^5	≥ 106
3	1.4×10^6	100–300
4	1.7×10^6	100–120
5	2.6×10^6	100–203
6	2.8×10^7	290–680
7	3.4×10^7	100–330
8	6×10^7	> 265

These results demonstrated an absence of detectable transfer of pBR322 to endogenous aerobic bacteria despite the 6-day survival of D20-5 in relatively large amounts. If transfer had occurred, we would have expected to isolate transfer-proficient derivatives of χ2236 and D20-5 and to have seen evidence of the plasmid in other *Enterobacteriaceae* among which transfer readily occurs in vivo and in vitro. The use of antibiotics in the media should also have enriched for any transfer that occurred, even at low frequency.

Our findings are relevant to the use of *E. coli* in recombinant DNA technology. Recovery of χ1776 in both humans and mice was limited to the first 24 hours af-

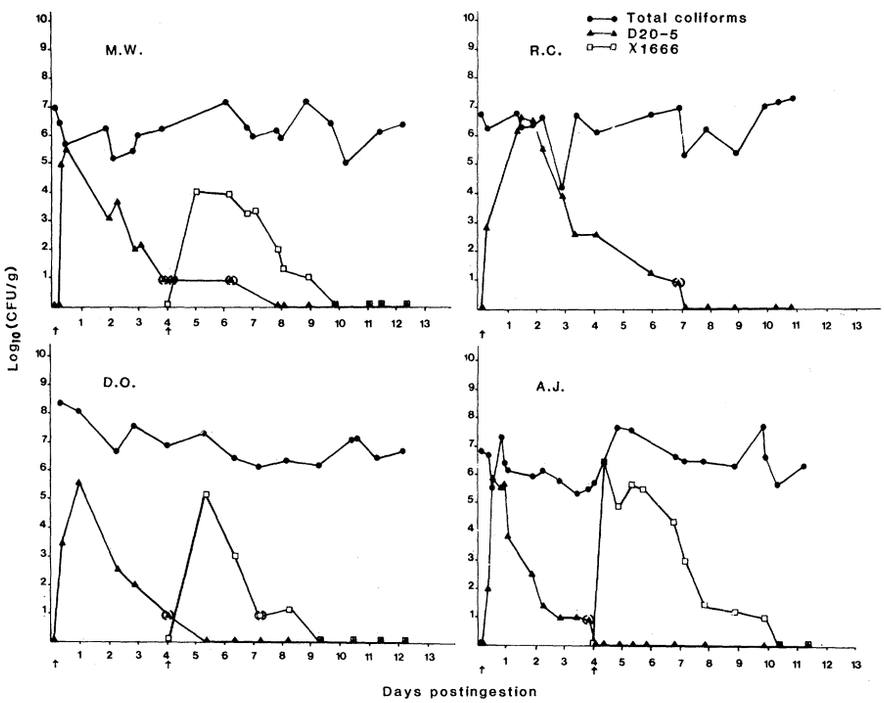

Fig. 3. Survival of χ1666 and D20-5 after the ingestion of 8.4×10^9 and 9.4×10^9 viable cells, respectively. Arrows indicate times of ingestion. Samples were diluted and plated on MacConkey agar with nalidixic acid (50 μg/ml) for χ1666 and the same media with tetracycline (10 μg/ml) and ampicillin (50 μg/ml) for D20-5. Symbols in parentheses indicate samples that contained organisms undetected on dilution plates, but that showed growth in broth (1 g/100 ml).

mice was limited to the first 24 hours after ingestion. In man, but not in mouse, χ2236 was recovered for 4 days. One reason for the difference may relate to the different diet and intestinal flora of mouse and man. It appears that the plasmid enhanced survival in this disabled strain as well as in the nondisabled χ1666 strain. Our data do not preclude survival of χ1776 below the detectable level. Survival of χ1776 with its plasmid was still 1/10,000 that of the wild-type *E. coli* K-12. Neither *E. coli* strain was able to colonize the intestinal tract of the volunteers, and neither was detectable more than 6 days after ingestion. During 4 to 6 days in the intestinal tract, the plasmid remained nonmobilized, at least by available detection techniques.

The survival of χ2236 was unexpected and raised questions about the effective biologic containment of this system. The failure to detect transfer of the plasmid to endogenous hosts reaffirmed the biologic containment of the EK2 system. Furthermore, no transfer was detected from the EK1 system despite survival at higher titers and for longer periods of time. These results, which provide data on survival of an EK1 and EK2 system in mammals, support their safe use in recombinant DNA technology under routine laboratory conditions. These studies raise the possibility, however, that other plasmid vectors may also enhance survival of the bacterial host. However, as long as plasmid vectors are selected for their inability to be mobilized, this possibility should not pose a problem.

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7. R. Curtiss III provided the χ1776, χ2236, and χ1666. The D20-5 was prepared by transforming χ1666 with pBR322 DNA obtained from G. Sutcliffe.
8. Composition per liter: peptone (10 g), dipotassium phosphate (2 g), agar (15 g), eosin (0.4 g), methylene blue (0.065 g), lactose (10 g), NaCl (5 g), yeast extract (1 g), diaminopimelic acid (100 μg/ml), thymidine (20 μg/ml), biotin (1 μg/ml), and nalidixic acid (50 μg/ml).
9. Isolates from each volunteer were sent to J. Donch (Palo Alto), who used biochemical tests to confirm the identity of each isolate with the host χ1776, and to R. Clowes (Dallas), who used plasmid isolation and restriction enzyme analysis to determine that pBR322 was the only plasmid in the recovered organisms.
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24 March 1980

Opiates and Opioid Peptides Hyperpolarize Locus Coeruleus Neurons in vitro

Abstract. *Intracellular recordings were made from locus coeruleus neurons in a brain slice preparation. Opiates and opioid peptides produced a dose-dependent, stereospecific, naloxone-reversible hyperpolarization of the neuronal membrane. This was associated with an increase in membrane conductance.*

The effects of opiates and opioid peptides on the firing rates of single neurons in the central nervous system have been widely documented (*1*). The most commonly observed response has been a decrease in the rate of neuronal firing. However, the extracellular recording techniques used could not elucidate the mechanisms by which the inhibition is produced nor whether it is mediated through a pre- or postsynaptic site. Therefore, to investigate the mechanisms underlying the inhibition of neuronal activity by opiates and opioid peptides, we recorded intracellularly from locus coeruleus neurons in vitro and studied the actions of these drugs on neuronal membrane properties.

The locus coeruleus is a homogenous group of catecholamine-containing cell bodies with projections throughout the central nervous system (*2*). This pontine nucleus provides an excellent site for studying the actions of opiates and opioid peptides, since it possesses a high density of opiate binding sites (*3*) and is innervated by nerve terminals that appear to contain enkephalin (*4, 5*). The locus coeruleus has been implicated as a site of opiate action because its destruction attenuates morphine-induced antinociception in rats (*6*). Previous electrophysiological studies of locus coeruleus neurons showed that opiates and opioid peptides depress both their spontaneous firing and the increase in firing following peripheral noxious stimulation (*7*). We now report that the application of opiates and opioid peptides to locus coeruleus neurons produces a stereospecific, naloxone-reversible membrane hyperpolarization that is associated with an increase in membrane conductance.

Thin slices of guinea pig pons were mounted in a recording chamber and superfused with Krebs solution at 37°C (*8*). Locus coeruleus neurons were impaled up to 9 hours after the slice was cut. Intracellular recordings were made from single cells for as long as 4 hours. The cells had stable resting membrane potentials of 45 to 72 mV. Drugs were applied to the tissue by changing the perfusion solution to one that differed only in its drug content.

Normorphine (100 n*M* to 3 μ*M*) caused hyperpolarization of the neuronal membrane in 19 of 28 locus coeruleus neurons tested (of the remainder, one was depolarized and eight were unaffected) (Fig. 1). The hyperpolarization was associated with a decrease in input resistance in 10 of the 14 neurons for which resistance measurements were obtained (Fig. 1, B and C). The degree of hyperpolarization was related to the concentration of the drug applied (Fig. 1A): at all concentrations tested, the effect began within 1 minute of exposure of the tissue to normorphine and was reversed when the tissue was washed with drug-free Krebs solution. The amplitude of the hyperpolarization produced by 1 μ*M* normorphine varied from 2 to 20 mV among neurons (mean, 9.8 ± 1.1 mV, $N = 19$), but for a given neuron the responses were reproducible. When neurons fired action potentials spontaneously, the hyperpolarization produced by normorphine was always associated with a marked reduction or abolition of the spontaneous firing.

The hyperpolarization of locus coeruleus neurons by normorphine was probably not the result of inhibition of the tonic release of an unknown excitatory neurotransmitter, because normorphine was still effective when the tissue had been perfused for up to 10 minutes with a solution containing no

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