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Proximity-Dependent Inhibition in *Escherichia coli* Isolates from Cattle▽

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We describe a novel proximity-dependent inhibition phenotype of *Escherichia coli* that is expressed when strains are cocultured in defined minimal media. When cocultures of "inhibitor" and "target" strains approached a transition between logarithmic and stationary growth, target strain populations rapidly declined >4 log CFU per ml over a 2-h period. Inhibited strains were not affected by exposure to conditioned media from inhibitor and target strain cocultures or when the inhibitor and target strains were incubated in shared media but physically separated by a 0.4-μm-pore-size membrane. There was no evidence of lytic phage or extracellular bacteriocin involvement, unless the latter was only present at effective concentrations within immediate proximity of the inhibited cells. The inhibitory activity observed in this study was effective against a diversity of *E. coli* strains, including enterohemorrhagic *E. coli* serotype O157:H7, enterotoxigenic *E. coli* expressing F5 (K99) and F4 (K88) fimbriae, multidrug-resistant *E. coli*, and commensal *E. coli*. The decline in counts of target strains in coculture averaged 4.8 log CFU/ml (95% confidence interval, 4.0 to 5.5) compared to their monoculture counts. Coculture of two inhibitor strains showed mutual immunity to inhibition. These results suggest that proximity-dependent inhibition can be used by bacteria to gain a numerical advantage when populations are entering stationary phase, thus setting the stage for a competitive advantage when growth conditions improve.

With the discovery of quorum sensing in the 1960s and 1970s, in comparison to the discovery of colicins in the 1920s, it became evident that populations of individual cells are capable of coordinating functions by using signaling molecules for communication. These communications can enhance fitness in a multispecies community, help exploit nutrients more efficiently, increase cooperation with neighboring cells, or harm competing bacteria. Some of the cell-to-cell communications have been well characterized. *Streptococcus oralis* 34 secretes an autoinducer that signals *Actinomyces naeslundii* T14V to congregate with *S. oralis* 34 and form a mutualistic biofilm in saliva (21). *Vibrio cholerae* uses autoinducers to shut down expression of virulence factors and biofilm formation at high cell densities (13). As an SOS response, certain bacteria can produce toxins, called bacteriocins, which can kill a narrow spectrum of competing cells that express suitable cell surface receptors (8–10, 14). Bacteria can also produce inhibitory phage particles and iron-sequestering aerobactin to gain an advantage over competing bacteria (6, 23).

Many of these mechanisms enhance the fitness of bacterial strains in a given environment. Khachatryan et al. in 2004 observed a fitness trait allowing certain multidrug-resistant *Escherichia coli* in Holstein calves to dominate the enteric *E. coli* population (16). Neither antimicrobial drug use nor the presence of antimicrobial resistance genes was associated with the fitness trait observed in the multidrug-resistant *E. coli* in these animals (12, 16). A fitness advantage could be shown by direct competition studies *in vitro* (16), and a clear advantage was evident when a milk supplement was included in the calf diet (11). The mechanism by which the fitness advantage was conferred has not been identified for either *in vitro* or *in vivo* cases.

Two mechanisms could explain the fitness advantage of these *E. coli* strains, which is reportedly associated with resistance to streptomycin, sulfadiazine, and tetracycline (SSuT^r *E. coli*). These strains may be niche adapted and able to easily outgrow less-adapted strains (metabolic advantage), but it is not clear that such a mechanism would span *in vitro* and *in vivo* growth conditions (16). Strains could also have an advantage if they are able to modify their environment by producing toxins, bacteriocins, or related compounds that can directly inhibit competitors (6, 8–10, 14, 23). By using an *in vitro* competition model, we report here that the success of calf-adapted *E. coli* strains is not associated with detectable growth rate differences compared to less-competitive strains but rather is associated with the ability to inhibit competing strains by a mechanism that appears independent of soluble toxins, bacteriocins, and lytic phages. Close physical proximity is required for inhibition to occur. The inhibitory phenotype is most easily observed under nutrient-limiting conditions, when the inhibitor strain is in transition from log to stationary growth phase. The inhibition phenotype is effective against a diverse panel of *E. coli*, including *E. coli* O157:H7. Finally, strains expressing the inhibitory phenotype are immune to inhibition by other inhibitor strains.

MATERIALS AND METHODS

Strains used in this study. *E. coli* 25 (SSuT^r) and *E. coli* 264 (nonresistant to antimicrobial drugs) were originally identified by Khachatryan et al. (15) and were used here as representative inhibitor strains. Thirteen strains of *E. coli* were cocultured with the inhibitor strains, and these were designated "target" or "susceptible" strains for this study. These included three *E. coli* O157:H7 strains,

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TABLE 1. Mono- and coculture competition assay results with inhibitor strains and target strains after 24 h

Strain combination assayed	Mean $\log_{10}$ CFU/ml[a]			
	Monoculture		Coculture	
	Inhibitor	Target	Inhibitor or noninhibitor	Target[e]
Inhibitor[b]/target strain combinations				
E. coli 25/*E. coli* O157:H7 strain 1[c]	9.43	9.28	9.18	2.90
E. coli 25/*E. coli* O157:H7 strain 2[d]	9.43	9.00	9.11	4.23
E. coli 25/*E. coli* O157:H7 strain 3	9.43	8.04	8.87	5.53
E. coli 25/*E. coli* 195	9.43	9.15	8.66	4.41
E. coli-25/*E. coli* 186	9.43	9.08	8.88	3.66
E. coli 264/*E. coli* 178 (SSuT^r)	9.15	9.15	8.83	5.32
E. coli 264/*E. coli* 4 (SSuT^r)	9.15	9.28	9.00	5.11
E. coli 264/*E. coli* 6 (SSuT^r)	9.15	9.26	9.00	4.40
E. coli 264/ETEC F5 (K99) strain 1	9.15	8.90	8.90	3.79
E. coli 264/ETEC F5 (K99) strain 2	9.15	8.80	8.80	5.92
E. coli 264/ETEC F4 (K88) strain 1	9.15	9.88	9.04	4.88
E. coli 264/ETEC F4 (K88) strain 2	9.15	9.11	9.04	2.48
E. coli 264/ETEC F4 (K88) strain 3	9.15	9.04	8.81	3.52
Noninhibitor/target strain combinations				
E. coli 178 (SSuT^r)/*E. coli* O157:H7 strain 1			9.26	8.49
E. coli 3211 (SSuT^r)/*E. coli* O157:H7 strain 1			9.15	7.90
E. coli 209 (SSuT^r)/*E. coli* O157:H7 strain 1			9.15	8.34
Inhibitor/inhibitor strain combination				
E. coli 25/*E. coli* 264			8.83[f]	8.76[g]

[a] Results are based on the mean log CFU/ml of a single experiment conducted with two technical replicates.
[b] *E. coli* 25 and *E. coli* 264 were the inhibitor strains used in this experiment.
[c] *E. coli* O157:H7 strain 1 was the prototype strain used in competition experiments.
[d] *E. coli* O157:H7 strain 2 (ATCC 700927).
[e] A statistically significant difference in counts was found for the inhibitor grown individually and in competition across all 13 strains, compared to that achieved by target strains (Student's paired *t* test, $P < 0.05$).
[f] Result for *E. coli* 25.
[g] Result for *E. coli* 264.

two antibiotic-susceptible *E. coli* isolates from dairy cattle, three SSuT^r *E. coli* isolates from dairy cattle, two enterotoxigenic *E. coli* (ETEC) isolates expressing F5 (K99) from cattle clinical samples, and three ETEC isolates expressing F4 (K88) from swine clinical samples (Table 1). Three SSuT^r *E. coli* isolates from dairy cattle that did not exhibit inhibitory properties were used as negative controls for competition experiments, and these strains were designated noninhibitor strains. With the exception of strain ATCC 700927 (*E. coli* O157:H7 strain 1), other strains were procured from the Washington Animal Disease Diagnostic Laboratory (Pullman, WA) and from the College of Veterinary Medicine Field Disease Investigation Unit (Pullman, WA). *E. coli* 93 (*cdiABI* positive) was kindly provided by David A. Low (University of California—Santa Barbara).

Nalidixic acid resistance was used as a selection marker for otherwise-antibiotic-susceptible isolates when in competition. Nalidixic acid-resistant mutants were selected after growing them in Luria-Bertani (LB) broth with increasing concentration of nalidixic acid over a period of 24 h. Colonies that were capable of growing on LB broth with nalidixic acid (30 μg/ml) were selected for subsequent experiments. Throughout these experiments, cell density was expressed as CFU per unit volume (ml) of medium, and CFU counts were estimated by dilution and spread plating on LB agar plates with appropriate antibiotics (nalidixic acid at 30 μg/ml; sulfadiazine at 500 μg/ml or streptomycin at 20 μg/ml).

***In vitro* competition assays.** Strains were initially streaked for isolation on LB agar plates with appropriate antibiotics, and overnight bacterial cultures were prepared by inoculating a single colony into 5 ml LB with no antibiotics. The inhibitor strain culture was mixed with the target strain culture, and this mixture was then added at either a 1:100 or 1:1,000 dilution into M9 minimal medium (6 g/liter Na_2HPO_4, 3 g/liter KH_2PO_4, 0.5 g/liter NaCl, 1 g/liter NH_4Cl, 2 mg/liter thiamine, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.2% glucose). Mixed cultures were incubated for up to 24 h at 37°C on a shaker (200 rpm).

E. coli 25, *E. coli* O157:H7, *E. coli* 264, and ETEC F4 (K88) strain 1 were used for detailed competition assays. All of these strains were initially grown individually in minimal medium, and aliquots for CFU enumeration were collected at 0, 2, 4, 6, 8, and 24 h. The strains were later paired to compete: *E. coli* 25 with *E. coli* O157:H7, and *E. coli* 264 with ETEC K88. Each strain was enumerated at 0, 2, 4, 6, 8, and 24 h. Results were averaged from three independent experiments.

In addition, inhibitor strain *E. coli* 264 was paired with several target strains, including ETEC K88 strain 2, ETEC K88 strain 3, SSuT^r *E. coli* 6, ETEC K99 strain 1, and *E. coli* 186 for competition. All of the competitions were conducted in minimal medium. Individual strains were enumerated at 0, 2, 4, 6, 8, and 24 h, and the ratio of target strain log CFU to inhibitor strain log CFU at each time point was determined.

Finally, inhibitor strain *E. coli* 25 or *E. coli* 264 was cocultured with 13 other *E. coli* strains of diverse origin. The strains were initially grown individually to determine growth curves, and then they were culture to compete in minimal medium and individually enumerated after 24 h of coculture. Noninhibitor strains (*E. coli* 178, *E. coli* 3211, and *E. coli* 209) competed as negative controls.

Conditioned media assays. Broth media of inhibitor and target bacterial cultures were collected and filtered (0.45-μm non-protein-binding syringe filters; Falcon, Franklin Lakes, NJ) to remove bacteria while allowing lytic phages or soluble proteins to pass. Filtrates were collected at mid-log (3 h) or late log (7 h) time points. An equal volume of filtrate was immediately added to a target strain broth culture in 2× medium, and the incubation was continued overnight. Stationary-phase filtrate was collected after 24 h of competition and added to cells grown for 3 h in 2× medium, and strains were individually enumerated 4 h later. This procedure ensured that nutrients were available for subsequent growth in the coculture experiments following the addition of stationary-phase conditioned medium filtrates.

As a positive control, when conditioned medium filtrate was collected after 7 h of coculture, individual strains were enumerated to confirm that inhibition was being expressed at that time. As a negative control, target strain cells were also enumerated after 7 h of growth in minimal medium alone (without addition of conditioned medium or inhibitor strains). Additional negative controls were included for enumeration of target cells after addition of conditioned media obtained from target cell broth cultures.

Inhibitor-to-target proximity assays. To determine if cell-to-cell proximity is required for inhibition, target strains *E. coli* O157:H7 and *E. coli* 186 and inhibitor strain *E. coli* 25 were grown in six-well plates with upper and lower chambers separated by polyethylene terephthalate (PET) membranes (Falcon, Franklin Lakes, NJ) as described previously (1). Two different pore sizes were used, including 0.4 μm (expected to block bacterial migration between wells) and 8 μm (expected to permit migration). The chambers were incubated at 37°C in a shaker incubator at 100 rpm, and individual strains were enumerated in both the upper and lower chambers. This experimental design, with 0.4-μm-pore-size membranes only, was repeated with the inhibitor strain *E. coli* 264 and target strain SSuT^r *E. coli* 6.

To confirm that the PET membranes were permissible to passage of lytic bacteriophages, we placed phage-susceptible *E. coli* strain DH10B in the bottom chamber and medium containing lambda phage GT10 (log 5.30 PFU/ml) in 2 ml of medium in the top chamber of 0.4-μm-pore-size membrane plates. The plates were incubated at 37°C for 5 h, and DH10B in the bottom chamber was enumerated. For negative controls, only medium was added to the top chamber.

Competition with *E. coli* 93 (*cdiABI*-positive strain). A contact-dependent mechanism for inhibition of *E. coli* was previously reported to involve the *cdiABI* gene, which encodes inhibition and immunity (1). *E. coli* 25 was competed with *cdiABI*-positive *E. coli* 93 both in minimal and LB media. Strains were enumerated at the start of competition (0 h) and after 24 h of coculture. To determine if this gene was present in inhibitors, PCR was used to detect the *cdiI* gene in *E. coli* 25 and *E. coli* 264, with strain EC93 serving as a positive control. Primers used were forward, 5′-GGCAAAATGAAGAAGAAACTATTTGCCC-3′, and reverse, 5′-ACAACAACTATTTTCTGTCTAAGATACTAAGGCC-3′), and DNA template was prepared by boiling lysis. Reaction mixtures contained 14 μl of CGS PCR master mix from Custom Genome Services (Pullman, WA), 0.5 μM primers, and 1 μl boiled lysate in 20-μl reaction mixtures. PCR amplification included an initial 2 min of denaturation at 95°C, followed by 28 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 45 s. As a positive control for amplification, the forward primer (5′-CGTAACCGTCTCGACAACATTATCTGG-3′) and the reverse primer (5′-TTTATCTGTCTGTACGGCAGTGAACATG-3′) for the gene *clpA* (ATP-binding subunit) was amplified (653 bp) successfully under the same PCR conditions.

Statistical analysis. The statistical analyses were performed by employing Student's *t* test and repeated measures analysis of variance (ANOVA) using the strain (dependent variable)-time (predictor variable) interaction (NCSS 2004 [Kaysville, UT] and Excel Microsoft software programs). A P value of <0.05 was considered statistically significant.

RESULTS

Inhibition occurs during the transition to stationary phase growth. *E. coli* 25 (SSuT^r) was used as a positive-control competitor for *in vitro* growth experiments with other commensal *E. coli* strains and is representative of the persistent SSuT^r *E. coli* strains observed in dairy calves (15). Wild-type *E. coli* 25 and *E. coli* O157:H7 strain 1 were used for detailed competition experiments. When the strains were grown individually in minimal medium, there was no significant difference between the log CFU of *E. coli* 25 and *E. coli* O157:H7 at the different time points used in the experiments (repeated measures ANOVA, $P > 0.05$) (Fig. 1, top graph). When cocultured, no difference in CFU was observed in the first 6 h of coculture (Fig. 1, bottom graph) ($P > 0.05$). However, a dramatic reduction in the *E. coli* O157:H7 count (Fig. 1, bottom) ($P < 0.0001$ for the strain-time interaction) was apparent at 8 h of coculture, a time when growth is in transition from log to stationary phase. After 24 h, *E. coli* 25 numbers had increased to >9 log CFU/ml, which was similar to counts observed when this strain was grown alone (Fig. 1, top and bottom graphs). However, the *E. coli* O157:H7 target strain counts remained low after 24 h (Fig. 1, bottom). Similar results were observed when inhibitor strain *E. coli* 264 was cocultured with ETEC F4 (K88) as a target strain (Fig. 1), with the only difference being that we observed inhibition by 6 h ($P < 0.0001$ for the strain-time interaction). Inhibitor strain *E. coli* 264 was then competed with five other target strains of diverse origins, and it similarly inhibited all of them within ~4 h of coculture (Fig. 2).

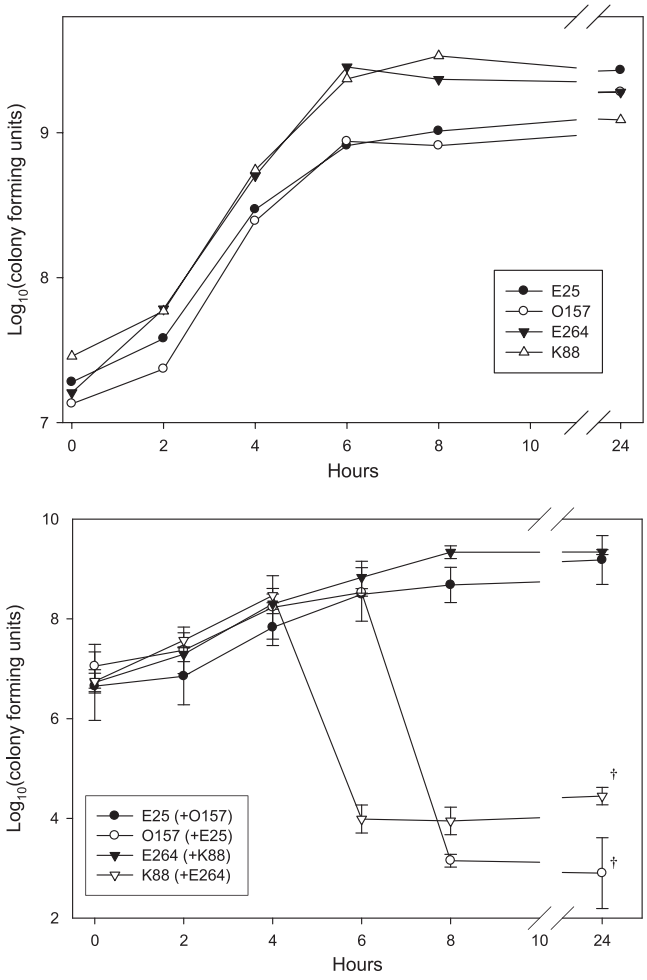

FIG. 1. (Top) Individual growth curves of inhibitor *E. coli* 25 (E25), inhibitor *E. coli* 264 (E264), target *E. coli* O157:H7, and target ETEC K88 in minimal medium. Standard errors, based on three independent experiments, each performed in duplicate, are not shown, in order to simplify the graph. (Bottom) Growth curves of inhibitor *E. coli* 25 (cocultured with *E. coli* O157:H7), *E. coli* 264 (cocultured with ETEC K88), target *E. coli* O157:H7 (cocultured with *E. coli* 25), and target ETEC K88 (cocultured with *E. coli* 264) in minimal medium. The bars indicate standard errors of the means and are based on three independent experiments, each performed in duplicate. †, $P < 0.0001$ (based on repeated measures ANOVA).

Inhibition is not caused by an accumulated soluble component in the medium. To determine if secreted toxin, bacteriocin, or lytic phage was limiting growth of target bacteria, we exposed actively growing target cells to conditioned media from inhibitor-target cocultures that were collected at different growth stages. Non-protein-binding 0.45-μm filters were used to sterilize the conditioned media. Filtrate was collected from growth stages before, during, and after the inhibitory effect, based on the inhibition kinetics of *E. coli* 25 and *E. coli* O157:H7 competition (Fig. 1, bottom). As a negative control, actively growing target cells were first exposed to conditioned media derived from target cells grown to early log (3 h), late

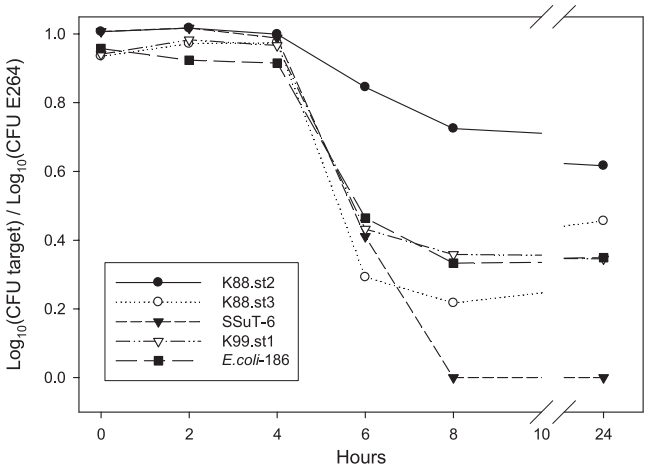

FIG. 2. Ratios of log CFU/ml of different target strains, including ETEC K88 strain 2, ETEC K88 strain 3, SSuT^r *E. coli* 6, ETEC K99 strain 1, and *E. coli* 186, to that of inhibitor strain *E. coli* 264 in minimal medium. Results are based on experiments performed in duplicate.

log (7 h), and stationary phase (24 h). After exposing the target cells, the counts were 9.35, 9.27, and 8.68 log CFU/ml, respectively. Similarly, when conditioned media were collected from cocultures of inhibitor and target strains, the media had not effect on the growth of target cells (9.39, 9.29, and 8.60 log CFU/ml, respectively). For the latter experiment we confirmed that inhibition took place by 7 h (target cocultured with inhibitor, 4.42 log CFU/ml; target grown alone, 8.70 log CFU/ml).

Proximity or direct contact is needed for inhibition. To determine if cell-to-cell proximity is required for inhibition, inhibitor and target cells were separated by porous membranes (0.4 μm or 8 μm) and the cells were enumerated in each chamber after 24 h of growth. Two competition experiments were conducted with the 0.4-μm filter, using *E. coli* 25 as the inhibitor strain in the top (experiment 1) or bottom (experiment 2) chamber with *E. coli* O157:H7 strain 1 and *E. coli* 186 used as target strains in the respective opposite chambers. The 0.4-μm-pore-size filter blocked movement of bacteria between the upper and lower chambers, as each strain was only detected in the chamber in which it was inoculated (Table 2). Both target and inhibitor strains grew to ≥9.0 log CFU/ml, indicating that there was no inhibition despite the potential for diffusion of soluble inhibitors across the membrane. When an 8-μm-pore-size membrane was used, target strain *E. coli* O157:H7 growth was significantly lower than the inhibitor strain ($P < 0.001$ in both experiments). Similar results were observed when *E. coli* 25 was cocultured with *E. coli* 186, with statistically significant inhibition of the target strain in both experiments ($P = 0.001$). Finally, inhibitor strain *E. coli* 264 was cocultured with target strain SSuT^r *E. coli* 6 in chambers separated by a 0.4-μm-pore-size membrane. There was no inhibition of the target cells, even when the inoculation chambers were reversed (Table 2).

To further verify that the membranes used in this study were permissible to diffusion of lytic phages, lambda phage was added to the upper chamber, with phage-susceptible *E. coli* strain DH10B in the lower chamber. Using 0.4-μm-pore-size membrane chamber plates, DH10B counts were significantly reduced when phage was added to the other chamber (4.3 log CFU/ml, versus 9.5 log CFU/ml without phage in the other chamber). These data demonstrate that the 0.4-μm-pore-size membrane was permissive to lytic phages. Assuming that soluble proteins were also not blocked by the 0.4-μm-pore-size membrane, we conclude that the inhibitor strains must be located in close proximity to or in direct contact with the target strains for inhibition to take place (Table 2).

A diverse panel of *E. coli* is susceptible to inhibition, but inhibitors are immune. To determine the spectrum of the inhibition toward other strains, we assembled a panel of *E. coli* isolates and confirmed their growth to >log 8.0 CFU/ml after 24 h of growth as monocultures in minimal medium. The panel included two inhibitor strains and 13 target *E. coli* strains (Table 1). Growth of susceptible target strains was significantly reduced during coculture with inhibitor strains compared with coculture with noninhibitor strains (paired *t* test, $P = 0.001$) (Table 1). Based on the CFU attained in monoculture for comparison, the mean reduction from coculture with inhibitor strains was 4.8 log CFU/ml (95% confidence interval, 4.0 to 5.5 log CFU/ml). *E. coli* O157:H7 strain 1 was also competed with three noninhibitory *E. coli* isolates, and this demonstrated that only a 1.4-log CFU/ml reduction can be expected from simple resource competition. In contrast, competition with inhibitor

TABLE 2. Competition results with inhibitor *E. coli* 25 and targets *E. coli* O157:H7 and *E. coli* 186 in chambers separated by a 0.4-μm or 8-μm membrane

Membrane pore size, expt no., and strain (chamber[a])	Mean log CFU/ml[b] after 24 h of incubation in:	
	Top chamber	Bottom chamber
0.4-μm membrane		
Expt 1		
E. coli O157:H7 (top)	8.95	Not detected
E. coli 25 (bottom)	Not detected	9.33
Expt 2		
E. coli 25 (top)	9.07	Not detected
E. coli 186 (bottom)	Not detected	9.34
Expt 3		
E. coli 264 (top)	9.37	Not detected
E. coli 6 (bottom)	Not detected	9.16
E. coli 6 (top)	9.30	Not detected
E. coli 264 (bottom)	Not detected	9.39
8-μm membrane[c]		
Expt 1		
E. coli O157:H7 (top)	4.75 ± 0.79[d]	4.41 ± 0.28[d]
E. coli 25 (bottom)	9.05 ± 0.16	9.41 ± 0.05
Expt 2		
E. coli 186 (top)	5.95 ± 0.29[d]	6.19 ± 0.13[d]
E. coli 25 (bottom)	8.72 ± 0.004	9.02 ± 0.06

[a] The chamber in which the strain was inoculated.
[b] The mean log CFU/ml data are from a single experiment with three technical replicates.
[c] Data for experiments with the 8-μm-pore-size membrane are the means ± standard errors of the means from three independent experiments each performed in duplicate.
[d] Statistically significant difference between counts of target cells and inhibitor cells in both the top and bottom chambers of growth ($P < 0.01$, based on Student's *t* test).

TABLE 3. Mono- and coculture results with inhibitor *E. coli* 25 and *cdiABI*-positive strain *E. coli* 93 in minimal medium and LB broth

Culture type, medium, and incubation time	Mean log CFU/ml[a]	
	E. coli 25	*E. coli* 93
Monoculture in minimal medium		
0 h	7.24 ± 0.02	7.29 ± 0.03
24 h	9.03 ± 0.02	9.03 ± 0.05
Coculture in minimal medium		
0 h	6.09 ± 0.01	6.06 ± 0.05
24 h	9.00 ± 0.01	4.03 ± 0.10[b]
Monoculture in LB medium		
0 h	7.31 ± 0.05	7.15 ± 0.01
24 h	9.38 ± 0.01	9.16 ± 0.07
Coculture in LB medium		
0 h	5.96 ± 0.10	5.99 ± 0.05
24 h	9.27 ± 0.09	6.04 ± 0.32[b]

[a] Results are means ± standard errors of means, based on three independent experiments each performed in duplicate.
[b] Statistically significant difference in the counts of *E. coli* 93 and *E. coli* 25 after 24 h of competition ($P < 0.01$, Student's *t* test).

E. coli 25 resulted in a 6.4-log CFU/ml reduction of *E. coli* O157:H7 strain 1 compared with monoculture. This indicates that the large majority of the reduction (>5.0 log CFU) is attributable to the presence of the inhibitor strain. When SSuT^r 25 and *E. coli* 264 were cocultured, both strains were immune to inhibition (Table 1). Taken together these data show that inhibitor strains inhibit the growth of susceptible target strains far more than what was expected from simple competition.

Competition of *E. coli* 25 and *cdiABI*-positive strain *E. coli* 93. To determine if the inhibitory mechanism observed in our experiments was distinct from a previously described *cdiABI*-mediated contact-dependent inhibitory mechanism (1), *E. coli* 25 was competed with a *cdiABI*-positive strain (*E. coli* 93) in minimal medium or in LB medium (1). Both of the strains were able to achieve >log 9.0 CFU/ml in minimal or LB medium when grown as a monoculture (Table 3). In coculture with *E. coli* 25, growth of *E. coli* 93 was reduced in both media (minimal medium, $P = 0.0001$; LB medium, $P = 0.0006$) (Table 3). *E. coli* 25 growth was not inhibited, achieving >9.0 log CFU after 24 h of coculture.

Strains *E. coli* 25 and *E. coli* 264 do not harbor the *cdiI* gene. Both the inhibitors were examined for the presence of the *cdiI* gene. While our PCR assay detected the predicted 252-bp gene fragment from strain *E. coli* 93, we were unable to amplify this gene fragment from our inhibitor strains. A PCR product from *clpA* (used as a positive control) was detected, and this confirmed that failure to amplify the *cdiI* gene fragment was not due to inhibition by impurities in the DNA template preparations.

DISCUSSION

Previous experiments had shown that SSuT^r *E. coli* exhibits a fitness advantage under both *in vitro* and *in vivo* conditions, but the underlying mechanism was not defined (11, 12, 16). To identify and characterize mechanisms responsible for these advantages, we conducted a series of pair-wise competition experiments using *E. coli* 25 against *E. coli* O157:H7 strain 1 as a target. Initial competition assays in LB broth indicated a slight growth advantage for *E. coli* 25 in a 24-h competition period (data not shown). When the same competition experiment was repeated in nutrient-poor minimal medium, however, we found that *E. coli* 25 became an extreme competitor, causing a large reduction of the target strain population (>4 logs) within a short period of time (<2 h). Target strain growth never recovered from this inhibition, even after overnight incubation. Presumably, the failure to recover is due to a lack of sufficient nutrients to allow the shift out of stationary phase.

In our coculture experiments, we observed that the inhibition occurs in mid- to late-logarithmic phase, although the exact timing was variable between cultures (Fig. 1, top and bottom graphs). We attribute this to normal experimental variation (e.g., inexact starting conditions). However, while the timing of inhibition was somewhat variable, the inhibitory phenotype was always expressed when inhibitor strains were cultured with target strains. The results were consistent and followed a similar pattern when strain *E. coli* 264 was used as an inhibitor (Fig. 1, bottom, and 2). Because the inhibition trait was expressed during the growth phase transition, we assessed the expression of inhibition at different densities and media conditions, but the results were inconclusive, and we were not able to decouple the confounding factors of density, growth phase, and nutrient availability (data not shown).

Nutrient-deficient conditions or overcrowding often trigger bacterial defense mechanisms that lead to the extracellular release of colicins, which can kill a narrow spectrum of nearby target cells that express suitable cell surface receptors (8–10, 14). We considered the possibility that the inhibitor strains studied here express a colicin that kills the target cells. However, we did not observe any decrease in inhibitor strain populations during the competition, as would be expected for bacteria producing group A colicins, which are also lethal to the producing cell (3). This observation does not rule out a role for group B colicins, which are not lethal for the producing cells (3, 22). Colicins are typically triggered as an SOS response when the cells enter stationary phase (4). We examined this question by exposing cells to mitomycin, which is known to induce an SOS response. The inhibition phenotype was unaffected regardless of when cultures were exposed to mitomycin (data not shown), and so we inferred that it is unlikely that proximity-dependent inhibition is part of an SOS response.

Based on our coculture graphs (Fig. 1, bottom graph, and 2), inhibitor and target strains are more likely to be transitioning from log to stationary phase at the time when the inhibitor phenotype is expressed. The pattern of inhibition was similar when different target strains were competed; inhibition was consistently detected in the transition phase. Furthermore, colicins are primarily encoded by plasmids (4). We failed to find any evidence of plasmids, based on plasmid analysis followed by purification with polyethylene glycol. A previous study also failed to find phenotypic evidence of colicins or plasmids in the *E. coli* 25 strain (15).

To account for any possible role of an extracellular inhibitory component, competing strains were separated using a 0.4-μm-pore-size membrane, which allowed the free exchange of media and soluble compounds, thereby exposing the target

strain to any extracellular components produced by the inhibitor strain while maintaining physical separation between the bacterial strains being tested. Separation with an 8-μm-pore-size membrane permitted migration of bacteria between wells, and there was a significant reduction in the counts of target strains. Provided that no soluble products were blocked by the 0.4-μm membrane, then the inhibitor bacteria must be in close proximity to or in direct contact with target cells for inhibition to occur. Another alternative explanation for the observed inhibition could be the presence of lytic phages (6, 23). Our phage control experiment confirmed that the 0.4-μm filter was permissible to passage of lytic phage, so the observed inhibition in this experiment was not due to the presence of a lytic phage from the inhibitor strains. From these experiments we observed no evidence of soluble inhibitory products or phages. We further showed that at the time when the target strain was being inhibited, conditioned media filtrates failed to inhibit the growth of target cells; this finding was also consistent with the conclusion that extracellular toxin, bacteriocin, or lytic phages are not involved in inhibition.

The requirement for close cell-to-cell proximity is not uncommon for cell signaling, but contact-dependent inhibition has only been identified recently (1, 17, 19). Aoki et al. (1) first observed contact-dependent inhibition in uropathogenic *E. coli* (EC93). In the case of EC93, two secreted proteins encoded by *cdiA* and *cdiB* (expressed in early logarithmic phase) bind to BamA in the outer membrane of target cells (*E. coli* K-12) to inhibit their growth. A protein encoded by a third gene, *cdiI*, protects the inhibitor strain from self-inhibition (2). PCR analysis of our inhibitor strains showed that they do not possess the *cdiI* immunity gene. However, it is possible that gene polymorphism may result in failure of PCR amplification. Therefore, we conducted competition experiments between *E. coli* 25 and strain EC93. Based on these assays, it is clear that *E. coli* 25 inhibits the growth of other strains by a different mechanism, since *E. coli* 25 was immune to inhibition by strain EC93 and, in addition, was able to inhibit the growth of EC93 in both minimal and LB media. Another proximity-dependent inhibition, called stationary-phase contact-dependent inhibition (SCDI), has been described for mutant *E. coli* K-12 strains. A single base substitution in a regulatory enzyme that catalyzes glycogen synthesis allows the mutant strains to kill or inhibit growth of nonmutant strains. This mutation also provides immunity for the mutant strain (17). A similar stationary-phase inhibition was observed in another mutant *E. coli* mutant in which a mutation in *uup*, a gene that encodes a soluble ATP-binding cassette, rendered the mutant strain sensitive to its parental strain when in close proximity (19). Both SCDI mechanisms result in inhibition that occurs in late stationary phase, after the cells have stopped growing, whereas proximity-dependent inhibition is detected in inhibitor *E. coli* culture when the cells are actively growing, in mid- to late log phase. Based on these characteristics, we propose that the mechanism of proximity-dependent inhibition is different from those previously reported. A precedent for the expression of an inhibitory mechanism that is nutrient dependent is the PrfA virulence regulatory protein in *Listeria monocytogenes*, which is upregulated in the presence of an intracellular carbon source (hexose phosphates) and downregulated in the presence of readily metabolized carbohydrates (5, 18, 20). A recently published study described a similar proximity-dependent inhibition mechanism between *Bibersteinia trehalosi* and *Mannheimia haemolytica* (7), but it is not clear if this mechanism is related to the *E. coli* mechanism described here.

A series of competition experiments were conducted to explore the scope of inhibitory activity, as most of the reported contact-dependent inhibition mechanisms have a narrow target range. *E. coli* that expresses P or S pili shows resistance to inhibition mediated by *cdiAB* (1), whereas the other two inhibitory mechanisms have only been described among mutant (versus parental) strains. Our inhibitory mechanism was effective against 13 diverse target strains, including pathogens (Shiga toxin-producing *E. coli* O157:H7, which causes bloody diarrhea and kidney failure in humans; ETEC expressing F5 (K99) fimbriae, which is responsible for neonatal diarrhea in cattle; and ETEC expressing F4 (K88) fimbriae, which is responsible for neonatal diarrhea in pigs. Some SSuT^r *E. coli* and antibiotic-susceptible commensal *E. coli* strains were also inhibited. The fact that the inhibitory trait was effective against pathogenic bacteria indicates potential therapeutic or prophylactic applications.

Proximity-dependent inhibition is consistent with previous findings showing a predominance of certain SSuT^r *E. coli* strains. In *in vitro* studies, Khachatryan et al. (16) competed SSuT^r *E. coli* with commensal *E. coli* in serial passage experiments and observed results consistent with the proximity-dependent selection described in the current study. For the *in vivo* studies conducted by Khachatryan et al., both SSuT^r *E. coli* and commensal-susceptible *E. coli* were grown to 8.0 log CFU/ml and mixed before inoculation into calves (16). These cell densities correspond to the values at which inhibition was observed in our experiments (Fig. 1, bottom, and 2). It is possible that target inhibition may have been initiated when cultures were mixed for inoculation or when these cells were exposed to the calf enteric environment, which may have provided the necessary trigger for inhibition leading to the predominance of SSuT^r *E. coli* over the course of the experiments.

Not much is known about the nutrient availability in the gastrointestinal tract and its influence on the growth and colonization of microbial species (5). Although the defined nutrient conditions in our study allowed us to effectively detect the expression of this inhibitory mechanism, we are not aware of how this phenotype is expressed in the intestinal environment. Nevertheless, if this phenotype is expressed within the enteric environments, then the proximity-dependent mechanism described here represents a potential means to gain a numerical advantage in an environment where resources are variable. For example, under favorable conditions (e.g., after food consumption), bacteria enter a growth phase, but as nutrients pass and become less abundant and the population begins to shift to a stationary phase, inhibitor strains could use proximity-dependent inhibition to "knock down" susceptible strains, providing a significant numerical advantage for the next time nutrients become available. It is possible that use of a dietary milk supplement that increased the relative abundance of SSuT^r *E. coli* in dairy calves (12) led to short-term population growth and early entry into stationary phase, when target cells are inhibited. If this happens on a daily basis, the inhibitor strains would have no difficulty dominating the *E. coli* population. To test these ideas, the genetic mechanism required for inhibition

needs to be identified and gene deletion and complementation studies are needed to clearly demonstrate the mechanism both *in vitro* and *in vivo*.

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REFERENCES

1. **Aoki, S. K., et al.** 2005. Contact-dependent inhibition of growth in *Escherichia coli*. Science **309:**1245–1248.
2. **Aoki, S. K., J. S. Webb, B. A. Braaten, and D. A. Low.** 2009. Contact-dependent growth inhibition causes reversible metabolic downregulation in *Escherichia coli*. J. Bacteriol. **191:**1777–1786.
3. **Cascales, E., et al.** 2007. Colicin biology. Microbiol. Mol. Biol. Rev. **71:**158–229.
4. **Chang, D. E., et al.** 2004. Carbon nutrition of *Escherichia coli* in the mouse intestine. Proc. Natl. Acad. Sci. U. S. A. **101:**7427–7432.
5. **Chico-Calero, I., et al.** 2002. Hpt, a bacterial homolog of the microsomal glucose-6-phosphate translocase, mediates rapid intracellular proliferation in *Listeria*. Proc. Natl. Acad. Sci. U. S. A. **99:**431–436.
6. **Cursino, L., et al.** 2006. Exoproducts of the *Escherichia coli* strain H22 inhibiting some enteric pathogens both in vitro and in vivo. J. Appl. Microbiol. **100:**821–829.
7. **Dassanayake, R. P., et al.** 2010. Bibersteinia trehalosi inhibits the growth of Mannheimia haemolytica by a proximity-dependent mechanism. Appl. Environ. Microbiol. **76:**1008–1013.
8. **de Lorenzo, V.** 1984. Isolation and characterization of microcin E492 from *Klebsiella pneumoniae*. Arch. Microbiol. **139:**72–75.
9. **Eraso, J. M., M. Chidambaram, and G. M. Weinstock.** 1996. Increased production of colicin E1 in stationary phase. J. Bacteriol. **178:**1928–1935.
10. **Gillor, O., B. C. Kirkup, and M. A. Riley.** 2004. Colicins and microcins: the next generation antimicrobials. Adv. Appl. Microbiol. **54:**129–146.
11. **Hayrapetyan, S. S., H. G. Khachatryan, and U. D. Neue.** 2006. A detailed discussion of the influence of the amount of deposited polymer on the retention properties of polymer-coated silicas. J. Sep. Sci. **29:**801–809.
12. **Hayrapetyan, S. S., U. D. Neue, and H. G. Khachatryan.** 2006. Changes in the pore character of silicas caused by surface bonding and polymer coating. J. Sep. Sci. **29:**810–819.
13. **Higgins, D. A., et al.** 2007. The major *Vibrio cholerae* autoinducer and its role in virulence factor production. Nature **450:**883–886.
14. **James, R., C. N. Penfold, G. R. Moore, and C. Kleanthous.** 2002. Killing of *Escherichia coli* cells by E group nuclease colicins. Biochimie **84:**381–389.
15. **Khachatryan, A. R., T. E. Besser, and D. R. Call.** 2008. The streptomycin-sulfadiazine-tetracycline antimicrobial resistance element of calf-adapted *Escherichia coli* is widely distributed among isolates from Washington state cattle. Appl. Environ. Microbiol. **74:**391–395.
16. **Khachatryan, A. R., D. D. Hancock, T. E. Besser, and D. R. Call.** 2004. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl. Environ. Microbiol. **70:**752–757.
17. **Lemonnier, M., et al.** 2008. The evolution of contact-dependent inhibition in non-growing populations of *Escherichia coli*. Proc. Biol. Sci. **275:**3–10.
18. **Milenbachs, A. A., D. P. Brown, M. Moors, and P. Youngman.** 1997. Carbon-source regulation of virulence gene expression in *Listeria monocytogenes*. Mol. Microbiol. **23:**1075–1085.
19. **Murat, D., L. Goncalves, and E. Dassa.** 2008. Deletion of the *Escherichia coli* uup gene encoding a protein of the ATP binding cassette superfamily affects bacterial competitiveness. Res. Microbiol. **159:**671–677.
20. **Park, S. F., G. S. Stewart, and R. G. Kroll.** 1992. The use of bacterial luciferase for monitoring the environmental regulation of expression of genes encoding virulence factors in *Listeria monocytogenes*. J. Gen. Microbiol. **138:**2619–2627.
21. **Rickard, A. H., et al.** 2006. Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. Mol. Microbiol. **60:**1446–1456.
22. **Riley, M. A., and D. M. Gordon.** 1999. The ecological role of bacteriocins in bacterial competition. Trends Microbiol. **7:**129–133.
23. **Smarda, J., D. Smajs, H. Lhotova, and D. Dedicova.** 2007. Occurrence of strains producing specific antibacterial inhibitory agents in five genera of Enterobacteriaceae. Curr. Microbiol. **54:**113–118.