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Loss of Culturability of *Salmonella enterica* subsp. *enterica* Serovar Typhimurium upon Cell-Cell Contact with Human Fecal Bacteria

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Loss of culturability of *Salmonella enterica* subsp. *enterica* serovar Typhimurium has been observed in mixed cultures with anaerobic fecal bacteria under conditions that allow local interaction between cells, such as cell contact. A reduction of a population of culturable *S.* Typhimurium on the order of $\sim 10^4$ to 10^5 CFU/ml was observed in batch anaerobic mixed cultures with fecal samples from different human donors. Culturability was not affected either in supernatants collected at several times from fecal cultures, when separated from fecal bacteria by a membrane of 0.45-μm pore size, or when in contact with inactivated fecal bacterial cells. Loss of culturability kinetics was characterized by a sharp reduction of several logarithmic units followed by a pronounced tail. A mathematical model was developed to describe the rate of loss of culturability as a function of the frequency of encounters between populations and the probability of inactivation after encounter. The model term $F(S \cdot F)^{1/2}$ quantifies the effect of the concentration of both populations, fecal bacteria (*F*) and *S.* Typhimurium (*S*), on the loss of culturability of *S.* Typhimurium by cell contact with fecal bacteria. When the value of $F(S \cdot F)^{1/2}$ decreased below ca. 10^{15} $(CFU/ml)^2$, the frequency of encounters sharply decreased, leading to the deceleration of the inactivation rate and to the tailing off of the *S.* Typhimurium population. The probability of inactivation after encounter, *P*, was constant, with an estimated value of $\sim 10^{-5}$ for all data sets. *P* might be characteristic of the mechanism of growth inhibition after a cell encounter.

Bacteria in nature display social activities such as cooperation and/or competition with other species. The adult human gut houses a bacterial community containing thousands of species-level phylogenetic types typically dominated by two bacterial phyla (divisions), the *Firmicutes* and the *Bacteroidetes* (1). These two main phylogenetic types interact with each other and with the host to ensure the stability of the gut ecosystem (1).

The gut microbiota is a major luminal barrier against pathogenic bacterial colonization (2). It profoundly influences the gut barrier function, host immunity, mucin biosynthesis, epithelial proliferation, and bacterial pathogenesis (2–4).

Most of the identified pathogenic bacteria exclusion mechanisms associated with the gut microbiota are mediated by production of extracellular components, especially short-chain fatty acids, produced by the microbiota as end metabolites (3, 4). However, secretion and detection of extracellular molecules in the surrounding environment is not the only form of bacterial interaction. Recently, cell-cell contact between bacteria has been demonstrated to be required for some bacterial interactions (5–10).

Salmonella enterica subsp. *enterica* serovar Typhimurium has been reported to be effectively outcompeted by gut bacteria in healthy mice (11). The aim of this work was to investigate if, similarly, human gut bacteria could have an effect on the population dynamics of *S.* Typhimurium. Several experimental approaches have been carried out to investigate interactions between *S.* Typhimurium and human commensal gut microbiota. A mathematical model has been developed in order to test hypotheses integrating the observed results.

MATERIALS AND METHODS

Bacterial strain, fecal samples, and culture preparation. *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain SL1344 was maintained at −80°C. Three successive subcultures in fermentation medium, prepared as previously described (12) and incubated at 37°C for 24 h to reach stationary phase, were carried out immediately prior to the experiments.

Fecal samples were processed by homogenizing ca. 25 g of fresh fecal samples from healthy donors, diluted (1:10) in oxygen-free buffer containing 0.1 M phosphate-buffered saline (PBS) solution, pH 7.4, in a Stomacher 400 (Seward, United Kingdom) at 230 rpm for 45 s. Fecal samples were obtained immediately prior to experiments.

Culture conditions were set up by dispensing 270 ml of fermentation medium into vessels magnetically stirred and heated at 37°C by a Grant (Jencons-Pls, United Kingdom) circulating water bath. The pH of the medium was maintained in a range from 6.8 to 7.2 by a pH control Fermac 260 (Electrolab, United Kingdom). The medium was continuously sparged with O_2-free N_2 to maintain anoxic conditions. Vessels were then inoculated with 30 ml of processed fecal samples and/or 1 ml of *S.* Typhimurium inoculum, diluted when required in fermentation medium to obtain the targeted initial concentrations.

Inoculation protocols. The following inoculation protocols were used to set up mixed cultures: (i) fecal inoculum added first to the vessel and incubated for 24 h prior to the inoculation of *S.* Typhimurium; (ii) *S.* Typhimurium and fecal samples inoculated at the same time into the vessels containing fresh medium and at similar concentrations of ca. 10^6 CFU/ml; (iii) *S.* Typhimurium inoculated first into the vessels and incubated for 24 h in order to reach a maximum density of ca. 10^9 CFU/ml prior to the addition of the fecal inoculum; (iv) reinoculation of *S.* Typhimurium into a mixed culture prepared according to protocol i and incubated for 72 h after the first inoculation of *S.* Typhimurium.

Supernatant collection. Supernatants were collected from mixed cultures of *S.* Typhimurium and fecal samples prepared according to protocol i. At several sampling times the content of the vessels was centrifuged at 7,000 rpm for 20 min and the supernatant was filtered in vacuum-driven Stericup filter units (Millipore, United Kingdom) with 0.22-μm-pore-size filters in an oxygen-free cabin.

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Two-compartment culture system. A two-compartment culture system was set up by placing a closed container made from 0.45-μm-pore-size membrane (Millipore, United Kingdom) in a vessel containing 300 ml of a fecal culture incubated at 37°C for 24 h in oxygen-free conditions and maintaining the pH in a range from 6.8 to 7.2. A volume of 1 ml of *S.* Typhimurium inoculum was added to the cell-free medium diffused within the container, which had a volume of ca. 25 ml; 10 ml of *S.* Typhimurium inoculum was added outside the container in contact with fecal bacteria.

Mixed culture with inactivated fecal bacteria. Inactivated fecal bacterial cells were obtained by centrifuging 300 ml of a fecal culture incubated at 37°C for 24 h under oxygen-free conditions and pH 6.8 to 7.2. The supernatant was filtered (0.45-μm-pore-size membrane; Millipore, United Kingdom) and maintained at the standard incubation conditions during the cell inactivation process in order to be used later as medium for the coculture. Fecal bacterial cells were inactivated in sodium cacodylate buffer containing 2.5% glutaraldehyde at room temperature for 1 h. After 3 successive washes with fresh fermentation medium, cells were resuspended in 150 ml of the initial supernatant, which was a reduced volume to compensate for cell loss during the cell inactivation process. By assuming 50% cell loss, it was ensured that the concentration of inactivated fecal bacteria was sufficient to result in a high frequency of cell encounters with *S.* Typhimurium. The inoculum of *S.* Typhimurium was added to this medium to evaluate the effect of cell-cell contact with inactivated fecal bacterial cells.

The inactivation of fecal bacterial cells exposed to glutaraldehyde was checked by plating out samples on Wilkins-Chalgren agar (Oxoid CM619) for total anaerobic counts (incubated in anoxic conditions at 37°C for 72 h) and on nutrient agar (Oxoid CM3) for total facultative aerobic counts (incubated in oxic conditions at 37°C for 24 h).

Bacterial counts. Samples from the cultures were plated on Wilkins-Chalgren agar for total anaerobic counts (incubated in anoxic conditions at 37°C for 72 h), on nutrient agar for total facultative aerobic counts (incubated in oxic conditions at 37°C for 24 h), on MacConkey no. 3 agar (Oxoid CM115) for counting *Enterobacteriaceae* (in oxic conditions at 37°C for 48 h), on Slanetz and Bartley agar (Oxoid CM377) for counting Gram-positive cocci (in oxic conditions at 45°C for 72 h), on Rogosa agar (Oxoid CM627) for counting *Lactobacillus* spp. (in anoxic conditions at 37°C for 72 h), on XLD agar (Oxoid CM469) for *S.* Typhimurium counts (in oxic conditions at 37°C for 48 h), on BMS agar (13) for counting *Bacteroides* spp. (in anoxic conditions at 37°C for 5 days), on Beerens agar (14) for counting *Bifidobacterium* spp. (in anoxic conditions at 37°C for 5 days), and on Wilkins-Chalgren agar containing 8 μg/ml novobiocin (Sigma) and 8 μg/ml colistin (Sigma) for counting *Clostridium* spp. (in anoxic conditions at 37°C for 5 days).

Model description. The pattern of loss of culturability of *S.* Typhimurium in coculture with fecal bacteria is characterized by a rapid reduction of several decimal logarithmic units of the culturable population followed by a gradual tailing-off pattern. The hypothesis of the model is that the rate of loss of culturability of *S.* Typhimurium due to local interaction with fecal bacteria is dependent, through a scaling function $G(x)$, on the product of the frequency of encounters between the two populations, Z, and the probability of loss of culturability after an encounter, P, leading to the expression

$$\frac{dS}{dt} = -\left(r \cdot S + G\left(Z \cdot P\right)\right) \tag{1}$$

$$\frac{dF}{dt} = -r \cdot F \tag{2}$$

where S and F denote the concentrations (CFU/ml) of *S.* Typhimurium and anaerobic fecal bacteria at time t, respectively, and r denotes the rate of decay of the population in a batch culture in stationary phase, which is assumed to be equal for the two populations as suggested by experiments performed in this work; however, in other conditions they could exhibit different decay rates and require an extra model parameter. This decay rate accounts for the loss of cell viability in stationary phase as well as for the reduction in plate counts due to the aggregation of bacteria in clusters.

The number of encounters per volume and unit time, Z, is dependent on the concentrations (CFU/ml) of the two populations, *S.* Typhimurium (S) and anaerobic fecal bacteria (F), the cross section or effective encounter area ($\pi \cdot D^2$, with D denoting the average bacteria diameter), and the average velocity of bacteria (v), as follows:

$$Z = \frac{1}{2} \cdot \pi \cdot D^2 \cdot v \cdot S \cdot F \tag{3}$$

The factor ½ avoids counting each encounter twice. The cell diameter and the cell velocity were assumed to be constant. A cell diameter of 2 μm was derived from the average of cell length and width, and the cell velocity, 7 μm/s, was estimated as the average of the published bacterial velocities measured by using a three-dimensional particle tracking technique (15).

This theory assumes that cell encounter is required but not sufficient for cell inactivation. After cell-cell contact, the process leading to cell inactivation occurs with a certain probability, P.

The chosen scaling function, $G(x)$, was the function $G(x) = x^n$. If $n = 1$, equation 1 describes a first-order kinetics for the loss of culturability of *S.* Typhimurium by cell-cell contact with fecal bacteria. The resulting expression follows collision theory, and it can be rearranged to obtain the Arrhenius equation. However, this is not appropriate to describe the observed tailing pattern of loss of culturability of *Salmonella* spp. in mixed cultures with fecal bacteria. First-order kinetics implies that when the population of fecal bacteria has a decay rate close to zero, which is often the case, the inactivation kinetics of *Salmonella* spp. is practically log linear, which is in disagreement with the observed tails in the inactivation curves of *Salmonella* spp. in mixed cultures. First-order kinetics expressions and linear models in general are not capable of describing appropriately the behavior of complex biological systems (16). For $n > 1$, equations 1, 2, and 3 describe a nonlinear system of differential equations. Similar expressions have been used to describe predator-prey systems (16). In predator-prey models, the prey rate of consumption is dependent on the predator population whereas the predator rate of growth is dependent on the prey population, leading to nonlinear differential equations that generally cannot be solved. The interaction phenomenon observed in this work differs from a predator-prey system in that the fecal bacteria population, which could be identified with the predator, is not affected by *Salmonella* spp., which would be the prey.

The explicit solution for the equations 1 and 2 of the model can be found as follows.

The solution of equation 2 for an initial value $F(0) = F_0$ is equal to

$$F(t) = F_0 \cdot e^{-rt} \tag{4}$$

For $n > 1$, with an initial value of $S(0) = S_0$ and substituting in equation 1 the expressions in equations 3 and 4, equation 1 has the following solutions:

$$\left(S(t)\right)^{1-n} = \frac{n-1}{r\left(-2n+1\right)} C F_0^n e^{-nrt} + \left(S_0^{1-n} + \frac{1-n}{r\left(-2n+1\right)} C F_0^n\right) e^{-(1-n)rt} \quad r \neq 0 \sqrt{x+y}\sqrt[2]{x-y} \tag{5}$$

where $C = \left(\frac{1}{2} \cdot \pi \cdot D^2 \cdot v\right)^n \cdot p^n$

The value of the exponent, n, governs the transition from the phase of sharp decrease of the population caused by the encounters with fecal bacteria to the decay phase of a stationary population in batch culture. With our experimental data, the most suitable value for n was 1.5, i.e., $G(x) = x\sqrt{x}$.

Model parameter estimation and model simulation. Parameters r and P from equations 4 and 5 were estimated by fitting the model to experimental measurements.

The decay rate of the population in stationary phase, r, was fitted by linear regression using the logarithm of the explicit solution of equation 2

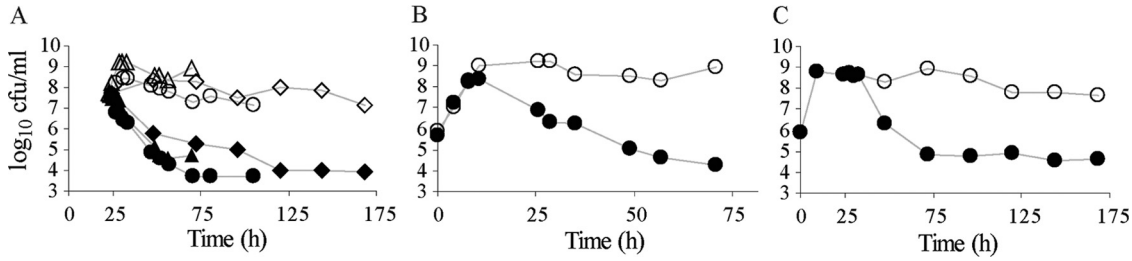

FIG 1 Comparison between the concentration of culturable *S.* Typhimurium (filled symbols) in anaerobic mixed cultures with fecal bacteria and the concentration of *S.* Typhimurium in pure cultures under identical conditions (empty symbols). Different inoculation protocols were assayed. (A) Fecal inoculum added to the vessels 24 h prior to the addition of *S.* Typhimurium. Different symbols show experiments set up with different fecal donors. (B) *S.* Typhimurium and fecal sample inoculated at the same time. (C) *S.* Typhimurium added to the vessel and incubated for 24 h prior to the addition of the fecal inoculum.

and the concentration of total anaerobic bacteria measured in time. The estimated value of r was constant and equal to ca. 0.01 h^{-1} in all assays.

The probability of loss of culturability after encounter, which is the parameter P in equation 5, was estimated by nonlinear regression using the measurements of the concentration of *S.* Typhimurium from the three data sets (see Fig. 5). An F test was used to test whether the fitted value of P differs significantly in any of the three data sets (16).

RESULTS

Reduction of the culturable population of *S.* Typhimurium in batch mixed culture with anaerobic fecal bacteria. A reduction of culturable *S.* Typhimurium in the order of approximately 10^4 to 10^5 CFU/ml was observed in cocultures with fecal bacteria from different human donors and several protocols of inoculation (Fig. 1). The concentration of *S.* Typhimurium in pure cultures under identical conditions did not show that reduction (Fig. 1). Donors were free of *Salmonella* spp. which were not detected when only fecal bacteria were inoculated (Fig. 2A). The magnitude of the difference in the logarithm of the concentration of *S.* Typhimurium when inoculated alone and in coculture with fecal bacteria is greater than 3 times the standard deviation of the observations, which has an average value of ca. 0.5 $\log_{10}$ CFU/ml, and further statistical assessment was not necessary. We use the terms growth inhibition or loss of culturability instead of killing or inactivation because *S.* Typhimurium cells lost the ability to form colonies on agar. In the experimental conditions of this work, nonculturable cells never recovered their ability to form colonies on agar. Whether viable but nonculturable bacteria are dormant or undergoing cell death is controversial (17).

The protocol of inoculation varied in the experiments shown in Fig. 1. In three experiments, fecal samples were incubated for 24 h prior to the inoculation of *S.* Typhimurium. The reduction of the population of *S.* Typhimurium was detected immediately after inoculation (Fig. 1A). When the fecal sample and *S.* Typhimurium were inoculated at the same time in fresh medium at similar concentrations, ca. 10^6 CFU/ml, both *S.* Typhimurium and fecal bacteria increased to reach a maximum concentration of ca. 10^9 CFU/ml in the first 12 h. This increase was followed by a sharp reduction of the culturable population of *S.* Typhimurium (Fig. 1B). When *S.* Typhimurium was inoculated 24 h prior to the fecal sample, the loss of culturability was detected ca. 12 h after the inoculation of the fecal sample (Fig. 1C).

The population kinetics of the main groups of fecal bacteria was not affected by the presence of *S.* Typhimurium (Fig. 2). In some of the experiments, inactivation kinetics similar to that observed in *S.* Typhimurium was observed in the population of total facultative aerobes as well as in the population counted on MacConkey agar selective for *Enterobacteriaceae* (Fig. 2). However, facultative aerobic bacteria of fecal origin did not always follow the same inactivation kinetics as *S.* Typhimurium and in some experiments their concentration did not decrease and maintained levels similar to that of total anaerobic bacteria (data not shown).

The loss of culturability of *S.* Typhimurium was detected in

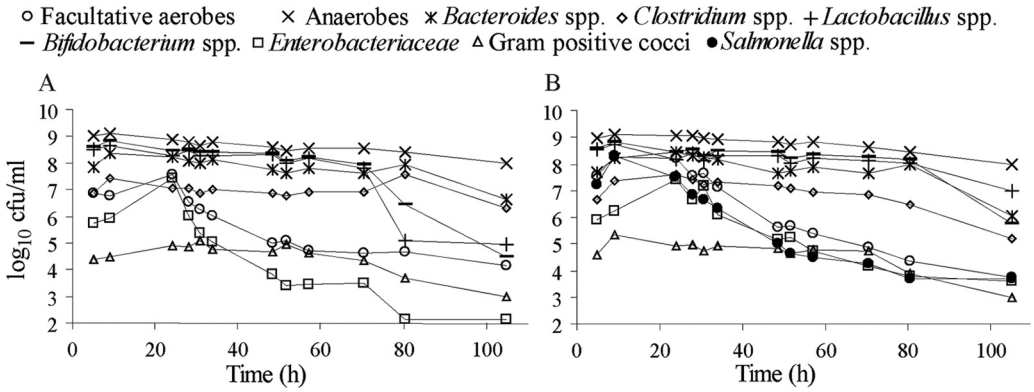

FIG 2 Population kinetics of the main bacterial groups in a culture inoculated with fecal bacteria only (A) and in a mixed culture of *S.* Typhimurium and fecal bacteria (B). Results are averages of 3 independent experiments inoculated with samples from different fecal donors. The standard deviation of the $\log_{10}$ CFU/ml ranged from 0.002 to 2, with an average of 0.5.

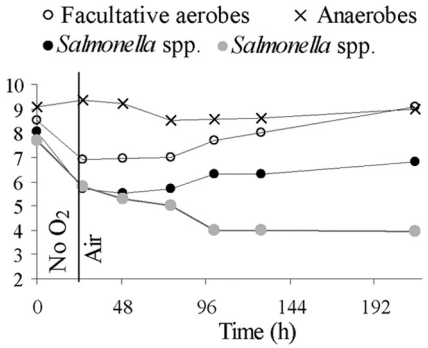

FIG 3 Population kinetics of *S.* Typhimurium and of fecal bacteria in a coculture maintained for the first 24 h under an anoxic condition and under an oxygen-rich condition afterwards. Gray symbols show *S.* Typhimurium counts in coculture with fecal bacteria under oxygen-free condition throughout the experimental period.

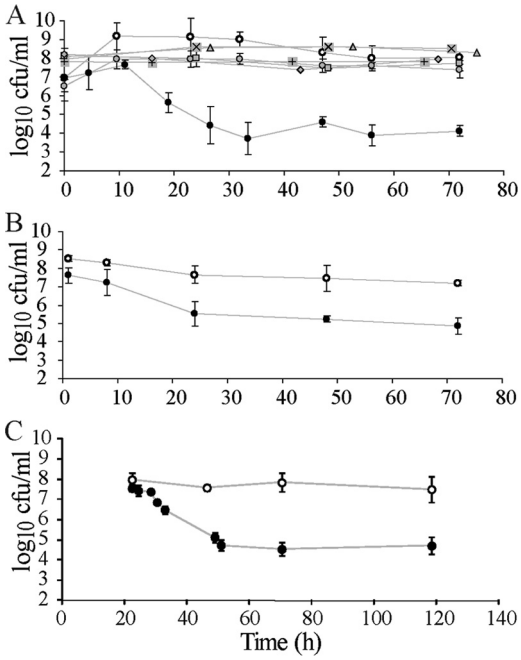

FIG 4 (A) *S.* Typhimurium concentration in supernatants collected anoxically from cocultures of *S.* Typhimurium and fecal samples after 4.5 (△), 11 (⊠), 19 (⧺), 26.5 (□), 33.5 (◇), and 72 (◯) hours of incubation. The concentration of *S.* Typhimurium was also monitored in a pure culture in fresh medium (○) and in a coculture of *S.* Typhimurium and fecal bacteria (●). (B) A closed container made of 0.45-μm-pore-size membrane was placed in a fecal culture in order to create a compartment with all soluble components but free of fecal bacteria. *S.* Typhimurium was inoculated into the fecal bacterium-free container (○) and outside the container (●) in contact with fecal bacteria. Results are averages of 4 independent experiments set up with samples from different donors. (C) Concentration of *S.* Typhimurium inoculated in medium containing inactivated fecal bacterial cells (○) and in coculture with living fecal bacterial cells (●). Results are averages of 2 independent experiments set up with different sample donors. All experiments (A to C) were carried out under oxygen-free conditions.

anoxic conditions only. The populations of *S.* Typhimurium and facultative aerobic bacteria decreased abruptly in mixed cultures in anoxic conditions; however the inactivation of both populations ceased when oxygen was introduced in the atmosphere. After 24 h under oxic conditions the number of fecal anaerobic bacteria decreased whereas facultative aerobic bacteria and *S.* Typhimurium increased (Fig. 3).

Loss of culturability of *S.* Typhimurium requires cell-cell contact with living anaerobic fecal bacteria. Supernatants were collected and processed under oxygen-free conditions from cocultures of *S.* Typhimurium and fecal samples at several sampling times during the inactivation of *S.* Typhimurium. After processing, supernatants were immediately reinoculated with *S.* Typhimurium. The concentration of *S.* Typhimurium was not affected in any of the supernatants, being not significantly different from that in fresh medium (Fig. 4A). Therefore, the inactivation of *S.* Typhimurium is not mediated by soluble components present in the supernatant of fecal cultures.

To prove further that the reduction in the population of *S.* Typhimurium requires local interaction with fecal bacterial cells, a closed container made from 0.45-μm-pore-size filter material was placed into a 24-h fecal culture. Soluble components could diffuse into the container, but cell trespassing was prevented by the membrane. *S.* Typhimurium was inoculated in the cell-free medium diffused within the container as well as outside the container, where cell-cell contact with fecal bacteria was possible. Thus, *S.* Typhimurium was exposed to nonprocessed fecal culture medium in communicated compartments that differed only by the presence of fecal bacterial cells in one of them. The concentration of *S.* Typhimurium in contact with fecal bacteria decreased abruptly in the first 24 h of incubation, whereas this decrease was not observed in the population of *S.* Typhimurium separated from fecal bacteria by the membrane (Fig. 4B). The degree of proximity between cells required for the loss of culturability of *S.* Typhimurium is not known. The terms "cell-cell contact" and "local interaction" are both used to described this requirement.

In addition, it was investigated if cell-cell contact in itself could be sufficient to cause loss of culturability of *S.* Typhimurium. To do this, the response of *S.* Typhimurium in contact with inactivated fecal bacterial cells was investigated. A fecal culture incubated for 24 h was centrifuged to collect both cells and supernatant. Fecal bacterial cells were inactivated and resuspended into the supernatant prior to the inoculation of *S.* Typhimurium. The results in Fig. 4C show that the culturability of *S.* Typhimurium was not affected by contact with inactivated fecal bacterial cells.

The loss of culturability of *S. Typhimurium* by local interaction with fecal bacteria is characterized by the probability of inactivation after cell encounter. The hypothesis to be tested was that the probability that an encounter results in loss of culturability, which is quantified by parameter *P* in the model, should be constant under identical experimental conditions. This hypothesis was supported by showing that the fitted value of *P* is the same for all data sets generated with several bacterial concentrations and inoculation protocols. Mixed cultures of fecal bacteria and *S.* Typhimurium were prepared at low, ca. 10^6 CFU/ml (Fig. 5A), and high, ca. 10^9 CFU/ml (Fig. 5B), concentrations. In addition, *S.* Typhimurium was reinoculated in a mixed culture 72 h after the first inoculation, when the initial population of *S.* Typhimurium was already tailing off (Fig. 5C). A value of ca. 10^{-5} for the probability of inactivation, *P*, was estimated by fitting the model with the three data sets (Fig. 5C). An *F* test showed that this value was not significantly

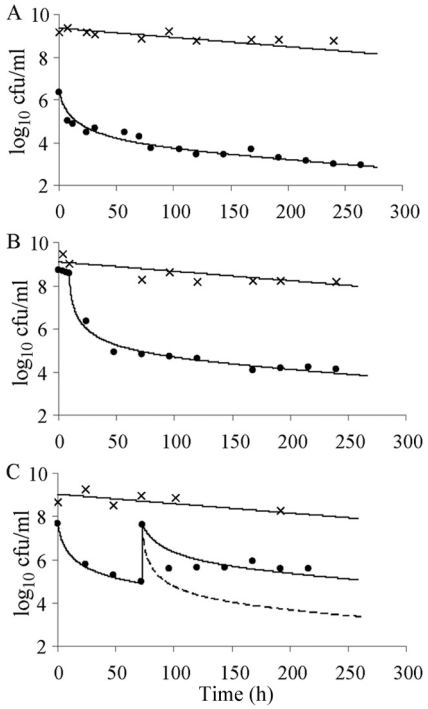

FIG 5 Model fittings (lines) obtained with the observed concentration of *S.* Typhimurium (●) and anaerobic fecal bacteria (×) generated with various protocols of inoculation. (A) Fecal inoculum added first to the vessel and incubated for 24 h prior to the inoculation of a relatively low inoculum, ca. 10^6 CFU/ml, of *S.* Typhimurium. (B) *S.* Typhimurium inoculated first into the vessels and incubated for 24 h to reach a maximum density of ca. 10^9 CFU/ml prior to the addition of the fecal inoculum. (C) *S.* Typhimurium reinoculated in a mixed culture with fecal bacteria 72 h after the first inoculation when the initial population of *S.* Typhimurium was already tailing off. Continuous lines represent predictions when the fecal population decays with a rate of -0.01 h^{-1}. The dashed line shows the predicted concentration of *S.* Typhimurium assuming that that the concentration of the fecal population does not change during the first 72 h of incubation.

different from those obtained when the model was fitted individually to each data set (P value = 0.63).

When the concentration of *S.* Typhimurium (Fig. 5B) in the mixed culture with fecal bacteria was high, the predicted and observed initial reduction of the population was larger and at a sharper rate, due to the higher frequency of cell encounters, than when the concentration of *S.* Typhimurium was smaller (Fig. 5A). When *S.* Typhimurium was reinoculated in a mixed culture in which the initial population of *S.* Typhimurium was already tailing off, both predictions and observations indicated that the reduction of the reinoculated population was smaller than that of the initially inoculated population of *S.* Typhimurium. This was due to a decrease of the frequency of cell encounters because of the relative slight decay of the anaerobic fecal bacteria during the course of the experiment. The fecal population decreased from ca. 10^9 CFU/ml to ca. 5×10^8 CFU/ml during the first 72 h preceding the reinoculation of *S.* Typhimurium. After the first inoculation with 10^9 CFU/ml fecal bacteria, a reduction of 2 decimal logarithmic units was detected in the population of *S.* Typhimurium in the first 24 h. However, a reduction of only 1.2 decimal logarithmic units was detected after reinoculation (Fig. 6C). Had not the fecal population decayed, a ca. 2-logarithmic-unit reduction would have been also expected after reinoculation (Fig. 6C).

The loss of culturability of *S. Typhimurium* by cell-cell contact with fecal bacteria is predicted as a function of the concentration of both populations. As indicated in equation 3, the frequency of cell encounters and thus the rate of loss of culturability were dependent on the concentration of both *S.* Typhimurium and fecal bacteria (Fig. 6A). For a concentration of fecal bacteria smaller than 6×10^6 CFU/ml, the model described here predicts that the loss-of-culturability rate of *S.* Typhimurium is practically equal to the decay rate of the population in stationary phase, $r = 0.01$ h^{-1}. When the concentration of *S.* Typhimurium is equal to 1 CFU/ml, more than ca. 10^{10} CFU/ml fecal bacteria are required to observe a loss-of-culturability rate significantly greater than 0.01 h^{-1}. Figure 6B describes the dependence of the rate of loss of culturability with the concentration of both populations. To predict loss of culturability of *S.* Typhimurium by local interaction with fecal bacteria, i.e., a rate significantly greater than the population decay rate in stationary phase, the product $F(S \cdot F)^{1/2}$, where F is the concentration of fecal bacteria and S of *S.* Typhimurium, must be greater than ca. 10^{15} CFU/ml^2 (Fig. 6B). This value is predicted assuming that the average bacterial cell velocity is equal to 7 μm/s. Bacterial cell velocity affects the frequency of encounters between the populations and thus the rate of loss of culturability of *S.* Typhimurium. To observe inactivation by cell-cell contact, the required concentrations increase when cells move more slowly and decrease for faster cell velocities. The required minimum value of $F(S \cdot F)^{1/2}$ is 10^{17} CFU/ml^2 when the cell veloc-

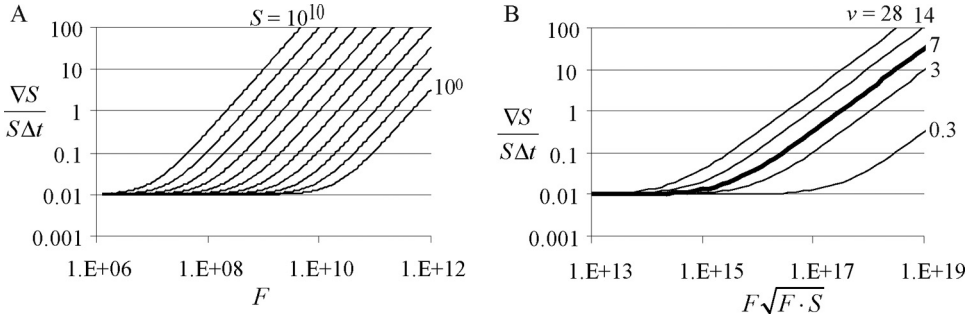

FIG 6 (A) Predicted loss-of-culturability rate of *S.* Typhimurium as a function of the concentration of fecal bacteria, F (CFU/ml), at several constant concentrations of *S.* Typhimurium, S, from 1 to 10^{10} CFU/ml. (B) Predicted relationship between the specific rate of loss of culturability of *S.* Typhimurium, $dS/(Sdt)$ (h^{-1}), and the concentration of *S.* Typhimurium, S (CFU/ml), and anaerobic fecal bacteria, F (CFU/ml), assuming several values for the average cell velocity, v, from ca. 0.3 to 30 μm/s.

ity is equal to 0.3 μm/s and 10^{14} CFU/ml when equal to 30 μm/s (Fig. 6B). These values define a range of velocities similar to those estimated experimentally by a three-dimensional tracking bacterial technique (15).

DISCUSSION

This study demonstrates the existence of a novel way of interaction between the gut microbiota and *S.* Typhimurium that requires cell contact or close proximity and leads to growth inhibition or loss of culturability of *S.* Typhimurium. The model presented explains the observed kinetics of the loss of culturability of *S.* Typhimurium as a function of the frequency of encounters between the two populations and the probability of inactivation after an encounter.

The preferential infection of the small intestine by *Salmonella* spp. is in part explained by the production of short-chain fatty acids by the gut microbiota. Formate is present at higher concentrations in the small intestine than in the colon and leads to the upregulation of invasion genes in *Salmonella* species (18). In contrast, butyrate, which is present at higher concentrations in the colon than in the small intestine, leads to the downregulation of invasion genes (4). An additional way of inactivation involving local interaction with the gut microbiota to prevent the colonization of the large intestine by *S.* Typhimurium is proposed in this work.

Contact-dependent bacterial interaction has been identified in strains of uropathogenic *Escherichia coli*, enabling them to inhibit the growth of other microbes in mixed populations (5). This phenomenon was mediated by the CdiA CdiB two-partner secretion proteins that bind to the outer membrane protein BamA in target cells (5, 19). This inhibitory process was dependent on the growth stage; exponentially growing bacteria but not stationary-phase cells were inhibitory while target cells were inhibited regardless of growth stage (5). In the work presented here, only stationary-phase cells of fecal origin were inhibitory to *S.* Typhimurium. When fecal bacteria were inoculated 24 h prior to the inoculation of *S.* Typhimurium, the reduction of the culturable population of *S.* Typhimurium was detected immediately after its inoculation (Fig. 1A). However, when the fecal sample was inoculated either simultaneously or after *S.* Typhimurium, an ~12-h delay was required to observe the reduction of the culturability of *S.* Typhimurium (Fig. 1B and C and Fig. 5B). The reason for this delay could not be an insufficient concentration of bacteria, because the product $F(S \cdot F)^{1/2}$ was greater than ca. 10^{15} CFU/ml^2, which is the threshold value predicted by the model developed here to observe loss of culturability of *S.* Typhimurium by cell contact. It has been reported that *E. coli* evolved in serial passage experiments and became able to kill or inhibit the growth of its own ancestors by cell contact only after reaching stationary phase (6). Growth inhibition by cell contact was suggested to be associated with mutations in the *glgC* gene and overproduction of glycogen (6) in non-growing cells of *E. coli* K-12. This strain lacks both *cdiA* and *cdiB* genes responsible for the growth inhibition process reported by Aoki et al. (5, 19). Thus, the initial delay that we observed in the loss of culturability of *S.* Typhimurium may be related to the time required by fecal bacteria to acquire those features that enable them to inhibit bacterial growth. An initial time delay following inoculation can also be observed in the inactivation of *E. coli* by cell contact with *Vibrio cholerae* mediated by the type VI secretion system (7). The type VI secretion system of *Pseudomonas aeruginosa* has also been reported to deliver two effector proteins, Tse1 and Tse3, in the periplasm of *Escherichia coli* and *Pseudomonas putida* by cell-cell contact causing cell lysis (9). An additional strategy suggested as a possible mechanism to deliver toxic molecules to neighboring cells is the formation of tubular extensions bridging cells or nanotubes (10). Therefore, cell-cell contact is essential for all these interactions, although the described molecular mechanism leading to loss of culturability, growth inhibition, or cell lysis varied between species.

Similar inactivation kinetics to that observed in *S.* Typhimurium was sometimes observed in the population of total facultative aerobes as well as in the population counted on MacConkey agar for *Enterobacteriaceae* (Fig. 2A and B). The similarity in the inactivation kinetics indicates that the loss of culturability of these bacterial groups could also require cell contact, as demonstrated for *S.* Typhimurium. This implies that loss of culturability upon cell-cell contact with fecal bacteria is not specific for *Salmonella* spp. and may also affect other species. Simple growth assays on selective media were carried out to follow the population dynamics of the main culturable fecal bacterial groups. For a thorough identification of microorganisms in fecal cultures, recently developed sequencing technology and metagenomic and bioinformatics methods are required (20, 21).

The loss-of-culturability kinetics observed in our experiments, as well as in other studies of local interactions leading to growth inhibition and/or inactivation (5–7), is characterized by a rapid reduction of several decimal logarithmic units of the culturable population followed by a gradual tailing-off pattern. In the model presented here, the rate of loss of culturability is dependent on the product of the frequency of encounters and the probability that after local interaction bacterial growth is inhibited. A significant loss of culturability is detected only if the value of $F(S \cdot F)^{1/2}$ is above a threshold that depends on the velocity of the cells. That threshold means that, for example, at an average cell velocity of 7 μm/s, when the population of *S.* Typhimurium is between 1 and 10^{10} CFU/ml, the required concentration of fecal bacteria is between 10^{10} and 10^{6} CFU/ml, in order to obtain a frequency of cell encounters sufficient for the inactivation to take place.

On the other hand, the hypothesis that the probability of loss of culturability after encounter is related to the mechanism of growth inhibition and therefore should maintain a constant value if estimated for the same local interaction process is supported by the results in this work. Indeed, a constant value for the probability of loss of culturability after cell encounter has been estimated for all experimental data sets generated with several concentrations of fecal bacteria and *S.* Typhimurium and various inoculation protocols. Thus, the probability of loss of culturability, *P*, could be the parameter characterizing the variety of mechanisms of growth inhibition and/or inactivation following cell encounter suggested in other studies (5–7, 9).

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