Antagonistic self-sensing and mate-sensing signaling controls antibiotic-resistance transfer

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Conjugation is one of the most common ways bacteria acquire antibiotic resistance, contributing to the emergence of multidrug-resistant "superbugs." Bacteria of the genus *Enterococcus faecalis* are highly antibiotic-resistant nosocomial pathogens that use the mechanism of conjugation to spread antibiotic resistance between resistance-bearing donor cells and resistance-deficient recipient cells. Here, we report a unique quorum sensing-based communication system that uses two antagonistic signaling molecules to regulate conjugative transfer of tetracycline-resistance plasmid pCF10 in *E. faecalis*. A "mate-sensing" peptide sex pheromone produced by recipient cells is detected by donor cells to induce conjugative genetic transfer. Using mathematical modeling and experimentation, we show that a second antagonistic "self-sensing" signaling peptide, previously known to suppress self-induction of donor cells, also serves as a classic quorum-sensing signal for donors that functions to reduce antibiotic-resistance transfer at high donor density. This unique form of quorum sensing may provide a means of limiting the spread of the plasmid and present opportunities to control antibiotic-resistance transfer through manipulation of intercellular signaling, with implications in the clinical setting.

During the past two decades, *Enterococcus faecalis*, a normal commensal in the intestinal tract (1), has emerged as a major nosocomial pathogen largely because of acquisition of genetic determinants for antibiotic resistance and virulence via horizontal gene transfer, especially through the efficient transfer of the conjugative pheromone-responsive plasmids, exemplified by pCF10 (2, 3). Donor cells carrying pCF10 are induced to high expression of conjugative transfer and virulence genes by a heptapeptide (LVTLVFV)-mating pheromone cCF10 (**C**) (4). Donor cells import **C** into the cytoplasm, where its binding to the pCF10-encoded master regulator Prg X [the protein product of *pheromone responsive gene X* (*prgX*)] abolishes repression of transcription of the *prgQ* operon encoding the conjugation genes (Fig. 1) (5, 6). The direct effect of **C** on *pheromone responsive gene Q* (*prgQ*) transcription is enhanced greatly by several co- as well as posttranscriptional mechanisms, whose cumulative effects cause the system to function as a bistable genetic switch (7). It also is noteworthy that pheromone induction increases the virulence of donor strains (8).

The mating response of donor cells to **C** is encoded by pCF10, whereas **C** is produced from a conserved chromosomal gene present in most if not all *E. faecalis* strains (4). To suppress self-induction by endogenously produced **C** in donors, a heptapeptide (AITLIFI) inhibitor molecule iCF10 (**I**) is encoded by the first gene in the polycistronic *prgQ* operon of pCF10 (9, 10). Both **C** and **I** are synthesized initially as prepeptides that, after cleavage of the leader sequence, are secreted into the extracellular environment as active peptides (9) (Fig. 1 and Fig. S1*A*). Once taken up by donor cells, **I** competes with **C** for binding to PrgX; the two peptides bind to the same subdomain of PrgX but have opposing effects on PrgX structure and function (11). It has been suggested that in the absence of recipient cells, the basal level expression of **I** keeps the expression of conjugation genes OFF in donor cells (11, 12). When recipient cells are in close proximity, the increased **C** level overcomes the inhibition of **I**, thus allowing donor cells to mount a mating response.

Although many studies of the pheromone induction process have been done, relatively little is known about the requirements for the return of the system to the "off" state following an induction cycle. In this study, we describe the results of experiments analyzing the turning off of conjugation, especially the role of **I** in the process. These results led to the discovery of the much broader role of **I** in the social behavior of donor cells, including its function as a unique quorum-sensing signal that inhibits conjugation. Our results suggest that these two antagonistic signals function in constraining the dissemination of the plasmid in mixed populations of donors and recipients. Manipulation of the newly described quorum-sensing circuit has potential as a therapeutic strategy to reduce resistance transfer and virulence in vivo.

Results

Pheromone Induction Generates a Burst of Transcription Through the *prgQ* Operon. Conjugative transfer of pCF10 is initiated by the induction of the *prgQ* operon that encodes **I** and the conjugative machinery required for plasmid transfer (Fig. 1). In uninduced cells, basal transcription from the P_Q promoter generates a 380-nt transcript, Q_S, which includes a single ORF (*prgQ*) encoding a 22-aa polypeptide processed into **I** and secreted into the growth medium (10). An increased intracellular level of **C**, resulting from addition of exogenous **C** or **C**-producing recipient cells, shifts the PrgX structure and oligomerization state from a repressing to a nonrepressing conformation, resulting in induction (11, 12). Induced donor cells contain increased levels of Q_S, as well as longer transcripts (Fig. 1), including the 530-nt Q_L and other mRNAs extending >10 kb into the operon (13, 14). These longer transcripts encode conjugation proteins (Fig. 1).

Because donor cells become competent for conjugation only when Q_L and longer *prgQ* transcripts are expressed (13–15), we examined the dynamics of turning ON and OFF of conjugation by using quantitative RT-PCR (qRT-PCR, Table S1) to measure levels of these transcripts following exposure to various levels of **C**. Q_L and transcripts of three downstream conjugation genes, *prgB, pcfC,* and *pcfG* (Fig. 1), showed similar rapid increases to maximum levels within 15–30 min of exposure of pCF10-containing donor cells to various amounts of **C** (Fig. 2 *A–D*). The expression of all four transcripts increased with increasing concentrations of **C**, but expression of all the genes began to

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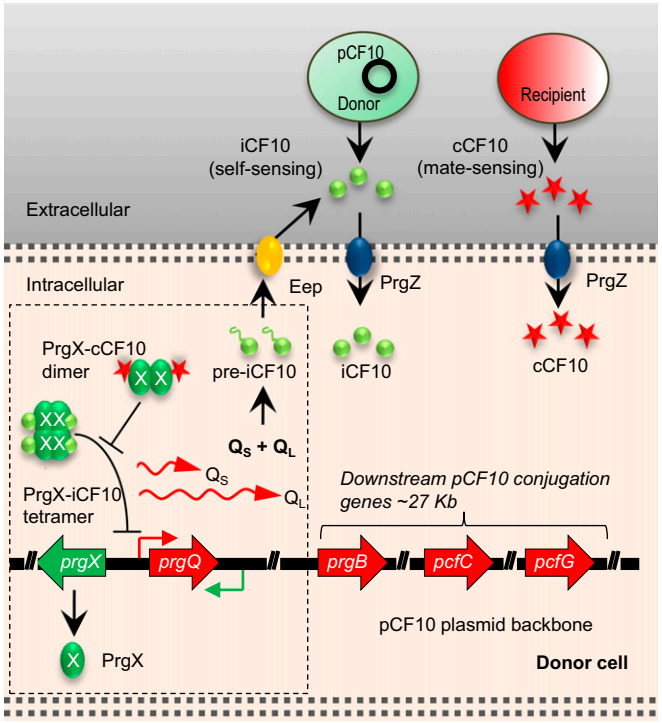

Fig. 1. Control of conjugation by two antagonistic peptide signals in *E. faecalis*. Signaling molecules cCF10 (**C**) and iCF10 (**I**) are released by recipient and donor cells, respectively, and imported into the donor cell, where these signals compete for binding to PrgX; PrgX negatively regulates the promoter for the *prgQ* operon, encoding conjugation functions. Whereas PrgX/**I** complexes repress transcription initiation, PrgX/**C** complexes do not. In uninduced donor cells, basal transcription of *prgQ* generates a short-transcript Q_S. Induced cells express higher levels of Q_S, as well as extended transcripts such as Q_L and other longer RNAs. Both Q_S and Q_L encode for the 22-aa preiCF10 polypeptide, which is processed into **I** and secreted into the growth medium. The opposing and partially overlapping *prgX* operon encodes PrgX protein and a small RNA Anti-Q that promotes termination of *prgQ* transcription. The unique organization of these two operons provides several layers of co- as well as posttranscriptional regulation that allow the system to function as a sensitive biological switch. In mixed cultures at low recipient cell density, the switch is off, whereas at high recipient cell density, sufficient **C** is produced to induce *prgQ* transcription, generating Q_L RNA and longer transcripts encoding downstream genes, including *prgB* (donor/recipient aggregation), *pcfC* (coupling of transferred DNA to the transfer machinery), and *pcfG* (relaxase, which nicks the plasmid DNA to initiate the transfer process).

decrease after 30 min. It is remarkable that all four genes showed a similar rapid return to basal expression levels following induction. The rapid turning off is not the result of a low level of inducer, as even at a saturation level of 50 ng/mL **C** (about 100× the concentration normally seen in mating experiments), the transcription was turned off after a short burst (4). The dynamic response after induction was also affirmed by RNA sequencing (Figs. S2 and S3) and Isobaric tag for relative and absolute quantitation (iTRAQ) (Figs. S4 and S5).

Mathematical Modeling of the Pheromone Response Dynamics. The fact that **I** first is secreted by donor cells then reimported to exert its conjugation suppression effect prompted us to hypothesize that **I** is a quorum-sensing signal (16, 17). To explore this notion further, we used a mathematical model describing the gene-regulatory circuit as well as population dynamics of donor, recipient, and transconjugant (recipient cells that acquire pCF10 by conjugation) cells (Fig. 1 and Fig. S1 *A*–*C*). We note that our previous modeling work reporting bistable switch characteristics of the *prgQ* operon in response to **C** (7) did not incorporate a role for **I** as a quorum sensor. In the current model, the concentration of **I** is allowed to vary, as in the case of changing donor cell density. The state of conjugation induction then is evaluated by the transcript level of Q_L (Fig. 2 *E* and *F*). The math-

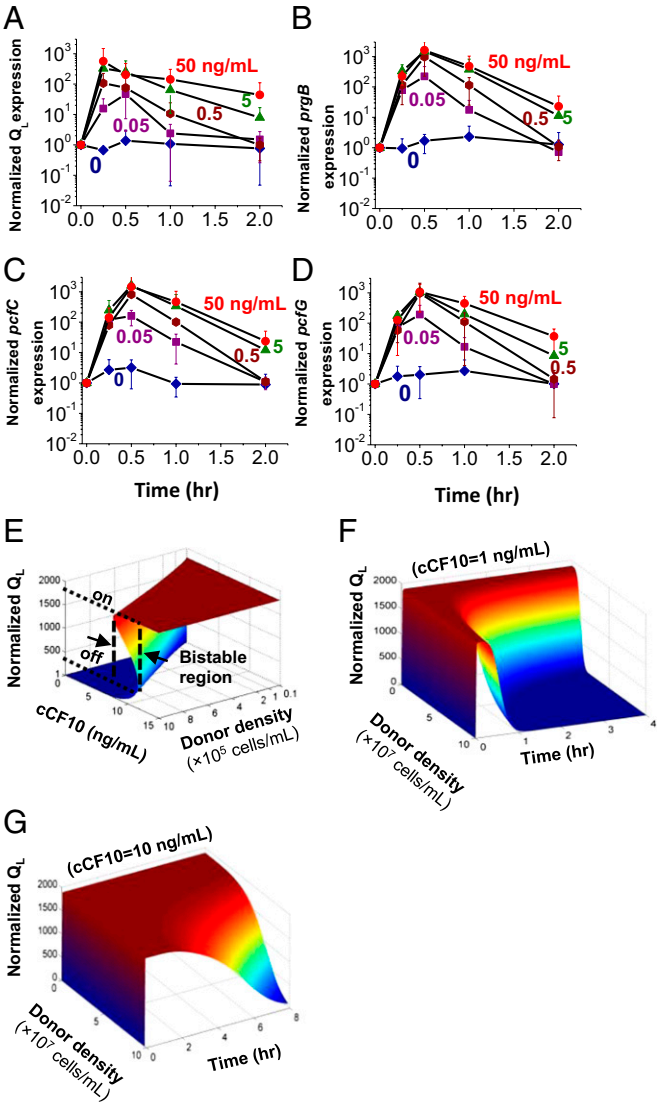

Fig. 2. Switch behavior of pCF10-carrying donor cells in response to pheromone induction. (*A*–*D*) qRT-PCR analysis of the dynamics of expression of conjugation determinants encoded by the *prgQ* operon in response to pheromone induction. mRNA was purified from cultures of pCF10-containing donors exposed to different concentrations of **C** for various periods. Transcript levels are shown for Q_L (*A*), *prgB* (*B*), *pcfC* (*C*), and *pcfG* (*D*), normalized relative to a transcript from a constitutive chromosomal gene, *gyrB*, and to time t = 0. Data shown in *A*–*D* are averages of three independent experiments (error bars are SDs from mean values). (*E*–*G*) Mathematical modeling of the pCF10-encoded pheromone response. (*E*) The steady-state response of Q_L (normalized to the off state) to induction demonstrates a characteristic bistable switch behavior. The bistable region shifts to a higher level of cCF10 with increasing donor density, accompanied by an increasing threshold level of cCF10 to turn on the conjugative genes. (*F* and *G*) Simulated dynamic response of Q_L transcript level (normalized to the off state at time t = 0) to **C** induction at various donor cell densities and at two different **C** concentrations: 1 ng/mL (*F*), and 10 ng/mL (*G*). At low donor density, Q_L is sustained at a high level over a longer period. At a high donor density, it decreases rapidly after a short period of high-level expression. At a high level of induction (10 ng/mL) corresponding to high a level of recipient cell density, Q_L stays high for a longer period.

ematical model incorporated the underlying genetic regulation of pCF10, which entails nonlinear interactions of sense:antisense transcripts within *prgQ* and *prgX* operons and interactions of PrgX with **C** and **I** (7, 11, 18) and is described in detail in *Supporting Information*. The parameter values were obtained from experimental results and from the literature (Table S2) (7).

The steady-state simulation showed a bistable switch-like response of Q_L RNA to induction with pheromone, over a wide range of donor densities. The bistable region, or the region in which two steady states coexist, changes with donor cell density (Fig. 2*E*). For donors originally at an off state and a cell density of 10^6 cells per milliliter, Q_L transcription remains at basal level (off) with increasing **C,** until **C** reaches 12 ng/mL; at that point, the steady-state transcript level of Q_L increases to that of an "on" state. On shifting down of donor cells from the on state, Q_L remains at an on level until **C** decreases to 8 ng/mL. As donor density increases, so does the concentration of **I**, causing the bistable region to shift toward a higher concentration of **C**; thus, a higher **C** concentration is required for donors to switch to an on state (Fig. 2*E*).

Upon induction, the rapid increase in *prgQ* transcription increases production of **I**, shifting the relative concentrations of **C** and **I**, and ultimately returning PrgX to the repressive conformation. At higher donor densities, the increase in **I** levels is faster. Donor cell density therefore also affects the duration of the on state: at a low densities, donor cells remained on for a long time (Fig. 2 *F* and *G*). At high densities, conjugation was turned off rapidly after induction (Fig. 2 *F* and *G*). As expected, the donor response also was influenced by recipient cell concentration, as illustrated by simulation at different **C** concentrations (Fig. 2 *F* and *G*). For any given donor cell density, a faster turning-off response was predicted at low recipient densities. Taken together, the model simulation results suggest that **I** is pivotal in regulating the turning off of conjugation and that **I** might function as a quorum-sensing signal of donor population density.

iCF10 Functions as a Quorum Sensor of Donor Population Density. Next, we carried out experiments to test the predictions of the model by examining the effects of donor density on the induction of conjugation. Fig. 3 depicts the results of mating experiments between donors and recipients in which the recipient population density was at the same high level in all cases but the donor populations were varied over a 100-fold range. The donors were uninduced initially, and the emergence of transconjugants was measured over time. In all three conditions, the appearance of transconjugants lagged for about an hour as **C** accumulated in the growth medium. At 3 h, the ratio of transconjugant to donor increased to 0.12 in matings with an equal number of recipients and donors. Remarkably, increasing the donor concentration by 10-fold suppressed the transfer of pCF10—no significant induction of transfer occurred—whereas in decreasing donor concentration to a recipient-to-donor ratio of 10, the number of transconjugants per donor increased. Thus, a high donor concentration had a suppressive effect on the conjugative transfer of pCF10 (Fig. 3).

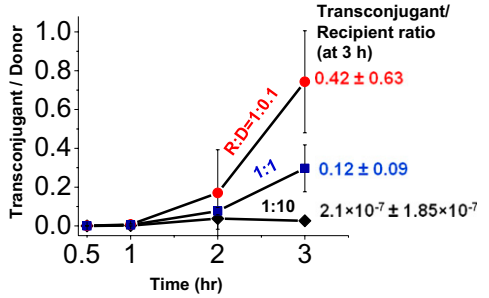

Fig. 3. Conjugation efficiency decreases at high donor densities. Recipient cells (~3.5×10^7 cells per milliliter) were mixed with different amounts of donor cells such that the recipient-to-donor ratios (R:D) ranged from ~1:10 to 1:0.1. Transconjugants, recipients, and donors were enumerated on selective agar medium after 0.5, 1, 2, and 3 h of coculture. The number of transconjugants formed per donor cell decreased with increasing donor density. Data shown are the average of at least three independent experiments (error bars are SDs from mean values).

The reduced efficiency of conjugation at a high donor-to-recipient cell ratio likely is the result of a faster turning off of induction. We analyzed the time dynamics of induction at high and low donor densities. Low-density donor cultures showed a stronger and more sustained response to **C** over a concentration range spanning 100-fold (Fig. 4*A*). In high-density cultures, the induction subsided rapidly, especially when the inducer **C** level was low (Fig. 4*B*). This is similar to the results shown in Fig. 2, which also were obtained at relatively high population densities.

We hypothesized that the rapid shut-off of the response at high density was caused by increased levels of **I** in the high-density cultures. We demonstrated this by evaluating the effect of **I** concentration directly. We constructed plasmid pCF10**I**dT, which is identical to pCF10, except it carries a deletion of the codon for the third amino acid residue of **I** peptide; this abolished **I** activity. When introduced into a wild-type host, the resulting OG1RF-pCF10**I**dT donor exhibited a de-repressed phenotype with a high Q_L expression because of the basal level of **C** production from the chromosome. Cultures of the mutant strain aggregated as the result of constitutive expression of PrgB and grew poorly unless a basal level of **I** was added exogenously (19). Upon induction with exogenous **C**, Q_L remained at the induced level in the absence of **I.** With a sufficiently high level of **I**, Q_L decreased rapidly (Fig. 4*C*). We then introduced pCF10**I**dT into JRC101, a host strain that does not produce **C**. The resulting donor strain did not express Q_L in the absence of exogenously added **C**, and the cells grew normally. Induction with 1 ng/mL of **C** caused Q_L to increase more than 400-fold. Q_L remained at the induced level in the absence or with a low level of **I** (1 ng/mL), but decreased rapidly when **I** concentrations of 10 ng/mL were added exogenously (Fig. 4*D*). A similar effect of exogenous **I** in suppressing Q_L after induction also was observed in a strain containing a mutation disrupting the Eep protease, which abolishes processing the pre**I** to produce mature **I** (Fig. S6).

These results strongly support the hypothesis that **I** is responsible for the suppressive effect of donor density on conjugation, acting both to interfere with induction and to increase the rate at which the system is shut off following induction. **I** thus functions as a classic quorum-sensing signal that allows donor cells to monitor their own density and calibrate their response to pheromone induction according to the relative abundance of recipients and donors.

Discussion

We demonstrate here that both the induction of conjugation and its subsequent shutting off in the *E. faecalis* sex pheromone response are affected by donor cell densities in an **I**-dependent fashion. We propose a model in which pCF10 conjugation is controlled by a dual cell–cell signaling system encompassing a traditional self-sensing signaling molecule (**I**) and a mate-sensing signaling molecule (**C**).

Quorum sensing in bacteria controls a wide variety of cellular processes (16, 17), including sporulation (20), competence (21), biofilm development (22), and virulence (23, 24). Quorum sensing is known to enhance conjugation in other bacteria, such as *Agrobacterium tumefaciens*, where an acyl-homoserine lactone signaling circuit activates conjugation at high cell density (25). In *E. faecalis*, we demonstrated the contrary, discovering a critical role for **I** as both an essential downstream effector in turning off

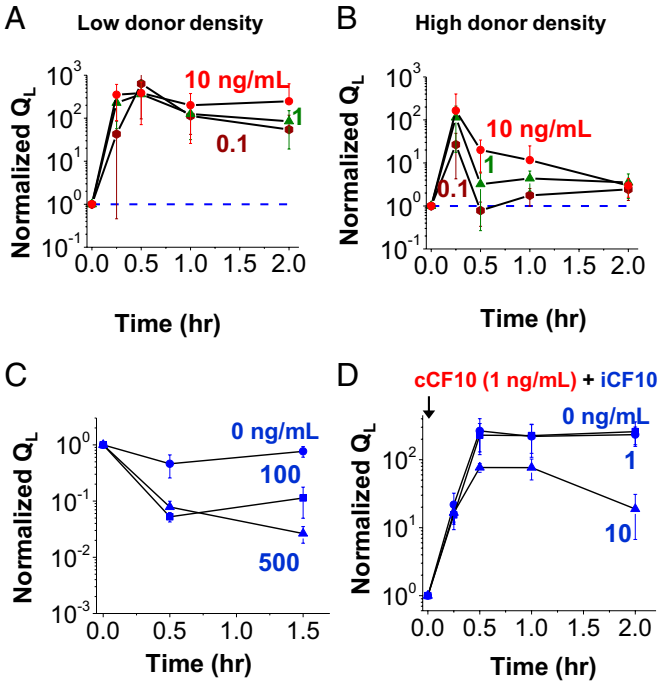

Fig. 4. **I** is responsible for the decreased pheromone response of high-density donor cultures and the return of donor conjugation to the off state. (*A* and *B*) The dynamics of turning on and turning off of conjugation in wild-type donor cells are altered by donor population density. Low-density (*A*) and high-density (*B*) cultures of wild-type donors were induced with various concentrations of **C**, and Q_L expression levels were measured by qRT-PCR. (*C*) Expression of Q_L in a donor with pCF10IdT. **I** was added at different concentrations. (*D*) Expression of Q_L in JRC101 carrying pCF10IdT. JRC101 does not produce **C**. **C** was added to induce conjugation gene expression. **I** was added at different concentrations along with **C**. At high **I** (10 ng/mL), the Q_L expression at the end of 2 h was significantly lower than at 1 h ($P = 0.049$), whereas at low **I** (1 ng/mL), Q_L expression did not decrease significantly compared with 1 h ($P = 0.279$).

the mating response to **C** and in blocking the ability of donors to initiate a mating response at high population density. Thus, in *E. faecalis*, quorum sensing has the ability to negatively affect conjugation frequencies.

At first glance, the existence of a plasmid-encoded product that hinders the proliferation of the plasmid seems counterintuitive if the plasmid is viewed as a "selfish" mobile genetic element evolved to disseminate via lateral transfer. Although the resistance and virulence genes carried by plasmids such as pCF10 may confer strong fitness advantages under selective conditions (e.g., during an opportunistic infection of an antibiotic-treated hospital patient), significant fitness costs likely are associated with pCF10 carriage under less selective conditions (e.g., during commensal intestinal growth in a healthy individual) in terms of plasmid carriage, expression of antibiotic resistance, and expression of conjugation genes (26). Replication and maintenance of the plasmid comprises some metabolic burden, and induction of a conjugation response requires the transcription and translation of more than 25 genes, as well as the assembly of a complex, ATP-dependent type IV secretion system to transfer the plasmid to recipients (27). Uncontrolled induction of conjugative genes not only is wasteful but also causes donor cells to aggregate because of the expression of PrgB protein. Whereas aggregation with recipient cells enhances mating, self-aggregation by donor cells at low recipient density only reduces growth rate and extracts extra metabolic costs. The calibrated response to both donor and recipient density in both turning on and shutting off of conjugative gene expression might provide a fitness advantage.

The self-sensing **I** signal also may play a role in restricting the spread of the plasmid in natural populations containing mixtures of donors and recipients. This would ensure that the population is endowed with antibiotic resistance while maintaining a subpopulation free from the metabolic burden of harboring a particular plasmid. Although this hypothesis requires further validation, the idea of a balanced, mixed population is supported by mathematical modeling (Fig. S7) and experimental evidence (Fig. S8) for the lack of complete conversion of recipients to donors in prolonged matings.

The dual signaling mechanism reported here may be rather common in nature, as it confers fitness advantages to the bacterial species in the face of multiple and sometimes opposing selective pressures. A new understanding of the interplay between the two signals may reveal means of interrupting its control circuit and offer new possibilities for suppressing virulence and resistance transfer in the clinical setting.

Materials and Methods

Bacterial Strains. The bacterial strains used in this study all were derived from *E. faecalis* strain OG1RF, whose complete genome sequence has been reported (28). In mating studies, OG1RFSSp was used as the recipient cell and strain OG1RF carrying plasmid pCF10 (denoted as OG1RF-pCF10) was used as the donor cell. Strain OG1RFΔEep, containing an in-frame deletion in the *eep* gene, and strain JRC101, in which **C** synthesis is rendered inoperative, were described previously (29). The construction of plasmid pCF10IdT, which produces an inactive **I** because of a T deletion, and the strains OG1RF-pCF10IdT and JRC101- pCF10IdT are described in detail in *SI Materials and Methods*.

Culture Conditions. All liquid cultures were grown in M9 medium containing 0.3% yeast extract, 1% casamino acids, 20 mM glucose, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$. For enumeration of bacterial populations, we used Brain Heart Infusion agar medium containing antibiotics selective for donors [rifampicin, 200 μg/mL; tetracycline (Tet), 10 μg/mL], recipients [spectinomycin (Spec), 1,000 μg/mL], and transconjugants (Spec, Tet).

To examine the expression of conjugation genes following **C** induction, overnight cultures of wild-type donors were diluted 1:10 and split into different tubes containing different concentrations of cCF10 and/or iCF10. Cell samples were collected at different time points, followed by the addition of RNAprotect Bacteria Reagent (Qiagen), and subjected to RNA extraction.

To examine the dynamic response of donor cells to pheromone (**C**) at varying cell densities (Fig. 4 *A* and *B*), overnight cultures of donors were spun down and washed once in 1 mL PBS containing 2 mM EDTA. Cells were diluted 1:10 (high donor density) or 1:1,000 (low donor density) into 4.5 mL M9 medium. Cultures were incubated for 1 h at 37 °C before cCF10 was added at different concentrations. Thereafter, the cultures were incubated on a shaker at 37 °C. Samples of cells were spun down, washed once in 1 mL PBS containing 2 mM EDTA. Samples were treated with RNAProtect Bacteria Reagent (Qiagen) before RNA extraction.

Mathematical Modeling. The mathematical model for conjugative transfer of pCF10 plasmid between *E. faecalis* donor–recipient cells consists of two main parts: a molecular model describing the regulation of conjugation of pCF10 in donor cells and a model depicting the interactions of donor and recipient cells. The first part of the model was described previously and shown to give rise to a bistable switch behavior (7). The previous model did not consider the effect of inhibitor **I** released by donor cells as a quorum-sensing signal. The original model was modified to allow for the consideration of the effect of donor cell density. The second part of the model considers growth of the donor and recipient population as well as the conjugation and conversion of recipient cells to donor cells. The molecular and population events depicted in the mathematical model are described in *Supporting Information* and shown in Fig. S1. The governing equations also are presented.

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