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Bacterial Conjugation in the Cytoplasm of Mouse Cells[▽]

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Intracellular pathogenic organisms such as salmonellae and shigellae are able to evade the effects of many antibiotics because the drugs are not able to penetrate the plasma membrane. In addition, these bacteria may be able to transfer genes within cells while protected from the action of drugs. The primary mode by which virulence and antibiotic resistance genes are spread is bacterial conjugation. Salmonellae have been shown to be competent for conjugation in the vacuoles of cultured mammalian cells. We now show that the conjugation machinery is also functional in the mammalian cytosol. Specially constructed *Escherichia coli* strains expressing *Shigella flexneri* plasmid and chromosomal virulence factors for escape from vacuoles and synthesizing the invasin protein from *Yersinia pseudotuberculosis* to enhance cellular entry were able to enter 3T3 cells and escape from the phagocytic vacuole. One bacterial strain (the donor) of each pair to be introduced sequentially into mammalian cells had a conjugative plasmid. We found that this plasmid could be transferred at high frequency. Conjugation in the cytoplasm of cells may well be a general phenomenon.

Since the advent of the antibiotic era, the use of antimicrobial agents has become routine and is currently widespread in clinical, veterinary, and agricultural (in both crop and animal husbandry) settings. Bacterial populations thus face great selective pressure to evolve resistance mechanisms and to disseminate resistance genes. Furthermore, antibiotic treatment has been shown to result in the overgrowth of resistant bacterial strains, as susceptible normal flora succumb to the effects of the drug. A hallmark of enteroinvasive pathogenic bacteria such as *Listeria*, *Salmonella*, *Shigella*, and *Yersinia* spp. is that they occupy an intracellular niche, such as vacuoles or the cytoplasmic space (14), thereby evading the effects of conventional antibiotics that are unable to penetrate the host cell membrane (19, 35, 42, 53). The cytoplasmic environment of the host cell is thus an antibiotic-protected niche that intracellular bacteria might employ for the transfer of resistance factors.

Virulence genes are also often found on horizontally mobile elements such as plasmids (20), and conjugation is a major mode of virulence gene transfer between bacteria. It has been shown that rates of conjugation and horizontal gene transfer are higher in a biofilm (47), which, like the cytoplasmic space, is also a niche that protects bacteria from antibiotics (10, 41). Importantly, biofilm bacteria may exchange virulence and antibiotic resistance genes in this protected environment.

Salmonella enterica serovar Typhimurium is able to exchange genetic information in human intestinal cells (20). In this case, the bacteria are confined within membrane-bound

vacuoles, which essentially serve as extracellular compartments since the bacteria do not have access to the cytoplasmic space. Most antibiotics are unable to penetrate vacuoles, and those that do are rendered ineffective or less effective in the endosomal compartments due, for example, to the lower pH in those compartments (46). For this reason, *S. enterica* strains are able to survive, multiply (62), and even conjugate (20) in these compartments. However, it is currently unknown whether conjugation can take place in the cytosol of mammalian cells. This issue is highly relevant to bacteria such as *Listeria monocytogenes* (3) and *Shigella flexneri* (58), extremely pathogenic bacteria that have developed strategies to evade the host defense mechanism of phagosomal killing by entering cells via the endocytic pathway and then escaping from the phagosome into the cytosol.

Using EcSf2a-3, an avirulent *Escherichia coli-Shigella flexneri* hybrid (2) that is capable of infecting mammalian cells, we show here that intracytoplasmic bacteria can conjugate in the cytosol of mammalian cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are described in Tables 1 and 2. IncP plasmid RK2 encodes conjugation factors and kanamycin (Km) resistance (Km^r) as well as ampicillin resistance (Ap^r) and tetracycline resistance (Tc^r) (52, 67). Plasmid pUZ8002 (J. W. Wilson and D. H. Figurski, unpublished data) is a derivative of pUZ8 (Km^r Tc^r) (26) that carries the *oriT1* mutation of an RK2 *oriT1* derivative. The *oriT1* mutation severely reduces pUZ8 self-transfer by several orders of magnitude ($<10^{-3}$). Wild-type pUZ8 self-transfer is similar to that of RK2 because it uses the same conjugation system found on RK2. The *oriT1* mutation reduces self-transfer but not mobilization. Since self-transfer is not eliminated, rare self-transfer events can be selected easily (60). Plasmid pGB2Ω*inv* (23) encodes bacterial invasin from *Y. pseudotuberculosis* and confers resistance to spectinomycin (Sp) (Sp^r). Consistent with data from previous studies, we found that invasin can convert noninvasive DH10B into highly penetrant bacteria. Plasmid pACYC184 confers resistance to chloramphenicol (Cm) (Cm^r) (11, 54).

Mammalian cell culture. HeLa human cervix epithelial adenocarcinoma cells (ATCC CCL-2), HepG2 human hepatocellular carcinoma cells (ATCC HB-8065), NIH 3T3 mouse embryonic fibroblast-derived cells (ATCC CRL-1658), and 143B human osteosarcoma-derived cells (ATCC CRL-8303) were cultured

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TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Genetic properties or relevant genotype or phenotype	Relevant description	Reference or source
Strains			
EcSf2a-3	The chromosome is a chimera of *E. coli* K-12 strain 395-1 (Δ*aroD* for attenuation) and *S. flexneri* 2a strain 256 with a *Shigella* virulence plasmid mutant [pWR100(*icsA/virG*)]; this strain is competent for invasion, which requires both chromosomal and plasmid virulence genes	*E. coli-S. flexneri* hybrid; harbors the *S. flexneri* pWR100 virulence plasmid; taken up rapidly by β1 integrin-expressing cells; maximal phagosomal escape; attenuated for safety; this strain can invade human cells and escape vacuoles, but because it has a mutation in *icsA/virG*, it is unable to move between cells	2
DH10B	F⁻ *araD139* Δ(*ara leu*)7697 Δ*lacX74 galU galK rpsL deoR endA1 nupG recA1 mcrA* Δ(*mrr hsdRMS mcrBC*) (φ80d*lacZ*ΔM15)	*E. coli* strain commonly used for molecular cloning; nonhybrid; lacks pWR100; slow uptake into cells; minimal phagosomal escape	22, 49
Plasmids			
RK2	IncPα Tra⁺ Kmʳ Apʳ Tcʳ	Transfers itself efficiently; donor plasmid	1, 31, 52
pACYC184	P15A rep Cmʳ Tcʳ	Nonconjugative plasmid in our experiments (completely unable to self-transfer and unable to be mobilized at a detectable level); marker for recipient bacterium	11
pEGFP-N2	pMB1 rep Kmʳ *gfp*⁺	Nonconjugative plasmid (completely unable to self-transfer and unable to be mobilized at a detectable level) used for its antibiotic resistance marker	Clontech[a]
pGB2Ω*inv*	pSC101 rep Spʳ *inv*⁺	Encodes invasin protein of *Y. pseudotuberculosis*, which enables bacteria to be taken up by β1 integrin-expressing cells	23
pUZ8002	IncPα Tra⁻ (*oriT1*) Kmʳ Tcʳ	RK2-like, pUZ8 derivative; inefficient self-transfer (<10⁻³ compared to the wild type); used as negative control for the self-transmissible (donor) plasmid	60; J. W. Wilson and D. H. Figurski, personal communication
pWR100	*S. flexneri* 5 strain M90T, IncFII (*ipa-mxi-spa*)⁺ *icsA/virG*	*S. flexneri* virulence plasmid, mutated to be a *virG* mutant to prevent cell-to-cell movement. Facilitates rapid and efficient phagosomal escape	24, 44

[a] See http://www.clontech.com/images/pt/dis_vectors/PT3053-5.pdf.

in Dulbecco's modified Eagle medium (DMEM) (catalog number 11995-065; Invitrogen) supplemented with 10% fetal bovine serum (FBS) (catalog number 26140-079; Invitrogen) and antibiotics (penicillin-streptomycin) (catalog number 15140-122; Invitrogen) at 37°C with 5% CO_2. Mouse LR5 macrophage cells (a kind gift of John Loike, Columbia University) were grown in RPMI 1640 (catalog number 11875-093; Invitrogen) supplemented with 10% heat-inactivated FBS and antibiotics. Antibiotics were removed from the culture medium 24 h before infections.

TABLE 2. Properties of bacterial strains used[a]

Bacterial strain (plasmid[s])	Selection used	Properties		
		I	E	C
DH10B(pACYC184)	Cmʳ	−	−	−
DH10B(pGB2Ω*inv*, pACYC184)	Spʳ Cmʳ	+	−	−
DH10B(pGB2Ω*inv*, pEGFP-N2)	Spʳ Kmʳ	+	−	−
DH10B(RK2)	Kmʳ	−	−	+
DH10B(pGB2Ω*inv*, RK2)	Spʳ Kmʳ	+	−	+
EcSf2a-3(pACYC184)	Cmʳ	−	+	−
EcSf2a-3(pGB2Ω*inv*, pACYC184)	Spʳ Cmʳ	+	+	−
EcSf2a-3(RK2)	Kmʳ	−	+	+
EcSf2a-3(pGB2Ω*inv*, RK2)	Spʳ Kmʳ	+	+	+
EcSf2a-3(pGB2Ω*inv*, pUZ8002)	Spʳ Kmʳ	+	+	−

[a] I, competence for invasion, i.e., presence of invasin plasmid pGB2Ω*inv*; E, competence for vacuolar escape; C, competence for self-transmissibility of the conjugative plasmid; +, high; −, low.

Bacterial infection of cultured cells. The infection protocol was based on a previously reported method (21). Mid-log-phase bacterial pellets were resuspended in phosphate-buffered saline (125 mM NaCl, 10 mM NaHCO₃, 1 mM NaH₂PO₄, 5 mM KCl, 2 mM MgSO₄, 1.8 mM CaCl₂, 10 mM HEPES, and 10 mM glucose [pH 7.4] [PSS]) (16). The numbers of bacteria in liquid culture were estimated by measurements of the optical density at 600 nm (1 optical density unit at 600 nm corresponds to 8×10^8 bacteria/ml), and this value was used to determine the desired multiplicity of infection (MOI) (target MOIs were 5 for LR5 cells; 5, 100, and 200 for HeLa cells; 1,000 for HepG2 and 143B cells; and 10, 50, 100, and 1,000 for 3T3 cells). The actual MOI used was determined a posteriori by plating serial dilutions of this suspension on appropriate selection plates.

Mammalian cells were cultured without antibiotics for 24 h prior to infection in DMEM containing 10% FBS (culture medium). On the day of infection, monolayers were washed once with PSS just before infection. We found that infection results were most consistent when only the middle two wells of six-well plates were used and when the cell monolayers were ~90% confluent. Bacteria resuspended in PSS to the desired MOI (typically between 50 and 100 for the 3T3 cells used in the bulk of the experiments) were spun onto the cells for 10 min ($720 \times g$, with no brake) in a plate centrifuge (catalog number 5804; Eppendorf), followed by incubation at 37°C. Infection was allowed to take place for various times, as indicated in the schematic of the protocols, and the monolayers were then washed three times with PSS. The infected cells were then transferred to 37°C with 5% CO_2 for 45 min to 6 h in medium containing gentamicin (Gm) (Medium+Gm) (DMEM, 20 mM HEPES, 10% FBS, 50 μg/ml Gm [catalog number 15710; Invitrogen]) to kill extracellular bacteria (18, 65).

To determine the efficiency of cellular entry, the cells were washed three times with PSS and then lysed with ice-cold PSS containing 0.1% Triton X-100 (catalog

number 07100; International Biotechnologies). The lysates were plated at various dilutions in duplicate onto LB agar (Miller formulation, catalog number BP1425-500; Fisher), selecting for the appropriate antibiotic markers carried by the invading bacteria (Km sulfate [catalog number K4000-5G], Cm [catalog number C-1919], and Sp [catalog number S-9007]; Sigma-Aldrich). The CFU obtained thus correspond to the numbers of bacteria that had gained entry into the cells (21). The number of bacteria per cell was calculated as follows: (CFU × dilution factor × volume of lysate)/number of host cells in the monolayer.

To determine the efficiency of vacuolar escape, we employed a chloroquine (Cq) protection assay. Cq is concentrated in endocytic vesicles (39) and can thus be used to distinguish between internalized bacteria that remain in vacuoles and bacteria that are able to exit the vacuole and gain access to the cytoplasmic space (21). Medium+Gm containing Cq diphosphate (catalog number C6628; Sigma-Aldrich) (Medium+Gm+Cq) (DMEM, 20 mM HEPES, 10% FBS, 50 μg/ml Gm, and 100 μg/ml Cq diphosphate) was added to the monolayers for 45 min following the infection phase, followed by three washes in PSS, lysis, and plating as described above. In some cases, the monolayers were treated with Cq prior to bacterial infection to ensure that the antibiotic was washed away effectively so as not to interfere with the subsequent bacterial invasion (optional Cq pretreatment) (see Fig. 2A). The percentage of vacuolar escape was calculated as follows: (number of bacteria recovered with Cq treatment/number of bacteria recovered in the absence of Cq treatment) × 100.

To detect conjugation events in the cytoplasm, two rounds of bacterial infection were performed (see Fig. 3A). First, donor bacteria were spun onto the cell monolayer and allowed to invade the cells. Medium+Gm and then Medium+Gm+Cq were added to kill extracellular and vacuolar bacteria, respectively. The monolayers were washed to remove the antibiotics, followed by a second round of infection with recipient bacteria. After the second round of Gm and Cq treatments, the infected cells were incubated for various lengths of time (1 h to 18 h) to allow conjugation to occur. The culture medium contained a reduced concentration of Gm (DMEM, 10% FBS, and 5 μg/ml Gm [O/N medium]) to prevent extracellular conjugation (in our experiments, the MIC of Gm was 0.2 μg/ml) (data not shown). Cq is toxic to mammalian cells over long periods (12), so in some cases, the cells were treated for a third time with Cq immediately prior to lysis to kill any remaining intravacuolar transconjugants (optional, Medium+Gm+Cq for 45 min) (see Fig. 3A). The cells were then washed three times in PSS, lysed, and plated as described above.

RESULTS

The relevant genotypes and properties of the bacterial strains and plasmids used in this work are shown in Tables 1 and 2. Briefly, we used bacterial strain EcSf2a-3, which is competent for both cellular entry and vacuolar escape into the cytoplasm, and control bacterial strain DH10B, which is competent for neither. EcSf2a-3 and DH10B were transformed with the following plasmids, as required: pGB2Ω*inv* (invasin-encoding plasmid), pEGFP-N2 (marker plasmid for bacteria; Km[r]), and pACYC184 (marker plasmid for recipient bacteria; Cm[r]). Plasmids RK2 (self-transmissible, conjugative plasmid; Km[r]) and pUZ8002 (RK2-like, pUZ8 derivative, deficient for self-transfer; Km[r]) were conjugated into these bacteria. Self-transfer of pUZ8002, although rare, can be selected easily (60).

EcSf2a-3 is deficient for cellular entry but retains cytotoxic functions. *Shigella flexneri* is able to induce its own phagocytosis by nonprofessional phagocytic cells, such as the epithelial cells of the colonic mucosa ("M" cells) and cultured epithelial cell lines (e.g., HeLa) (58), by expressing virulence genes encoded by plasmid pWR100. This plasmid is sufficient to confer the invasive phenotype upon noninvasive *Escherichia coli* in vitro (56). *S. flexneri* infection of macrophages leads rapidly to an apoptotic-like death as early as 1 h after infection (71). *S. flexneri* penetration of HeLa cells is also efficient: bacteria lyse the phagocytic membrane and lie free within the cytoplasm in minutes (58, 64), but unlike infected macrophages, infected HeLa cells do not undergo apoptosis (43). As opposed to

highly pathogenic *S. flexneri*, EcSf2a-3 is an avirulent hybrid bacterial strain carrying chromosomal genes from *E. coli* and *S. flexneri* and the virulence plasmid pWR100 from *S. flexneri* (2, 50). EcSf2a-3 infection of mouse LR5 macrophage cells led to apoptosis (data not shown), consistent with results using wild-type *S. flexneri* (71). While EcSf2a-3 has also been reported to invade HeLa cells (2), we found that its levels of cellular entry were too low (data not shown) to be of use in our experimental protocol. Thus, compared to *S. flexneri*, EcSf2a-3 appears to be deficient in cellular entry but to retain cytotoxic function when engulfed by professional phagocytic cells.

Invasin enables EcSf2a-3 and DH10B bacteria to enter cultured cells. The *Yersinia pseudotuberculosis* invasin protein (Inv) is necessary and sufficient for bacterial invasion into nonphagocytic cells that express a subset of β1 integrins (32, 33). Since EcSf2a-3 enters cells at an inefficient rate, we introduced the *Y. pseudotuberculosis* invasin-encoding plasmid, pGB2Ω*inv* (23), into EcSf2a-3 cells to see if we could enhance the rate of cellular entry. The experiment was carried out as described in the legend of Fig. 1A. Briefly, bacteria were spun onto mammalian host cell monolayers and incubated for 30 to 180 min before treatment with Gm to eliminate extracellular bacteria. After 45 to 360 min of Gm treatment, the infected host cells were lysed and plated onto LB agar containing the appropriate antibiotic(s) to assay for internalized bacteria. We found that pGB2Ω*inv* increased the rate of entry of EcSf2a-3 into 3T3 mouse embryonic fibroblasts by 3 orders of magnitude (Fig. 1B). Cellular entry rates of *E. coli* DH10B cells containing pGB2Ω*inv* were also increased by 2 to 3 orders of magnitude in mouse 3T3 cells (Fig. 1B) as well as in human HepG2 and 143B cells (data not shown). The engulfment rates of the two bacterial strains were comparable in the cell lines tested (Fig. 1B). Cellular entry increased with increasing multiplicities of infection (MOI) (Fig. 1B) but not with increasing infection time, holding the MOI constant (data not shown), implying that MOI is a key factor determining infectivity.

EcSf2a-3 escapes from vacuoles at a high rate. Cq is an antibiotic that is concentrated within endocytic vesicles (15, 39). The Cq protection assay is used to determine the number of bacteria that are able to lyse the phagosomal membrane and escape into the cytoplasmic space (21, 70). We performed this assay to determine the phagosomal escape efficiencies of our *E. coli* strains. The experiment was carried out as described in the legend of Fig. 1A, with an additional step to perform the Cq protection assay. Briefly, following treatment with Gm, the infected cells were incubated in Medium+Gm+Cq (Fig. 2A). EcSf2a-3 bacteria, but not DH10B bacteria, carry *S. flexneri* chromosomal genes and *S. flexneri* virulence plasmid pWR100, which encodes the "invasin plasmid antigens," i.e., products of the *ipa* gene cluster, necessary for phagosomal escape (5, 9, 44, 57, 59). One of the proteins encoded by the *ipa* locus, IpaB, acts as a membrane-lysing toxin, enabling bacterial escape from the phagocytic vacuole to the cytoplasmic compartment (29). Thus, we expected Ecsf2a-3(pGB2Ω*inv*), but not DH10B(pGB2Ω*inv*), to be able to escape from phagosomes of 3T3 cells into the cytoplasm.

Both DH10B and EcSf2a-3 containing pGB2Ω*inv* gave comparable engulfment rates in 143B and 3T3 cells: in Fig. 1B, the efficiencies of cellular entry for the two bacterial strains were comparable for a given MOI and similarly in Fig. 2B, compar-
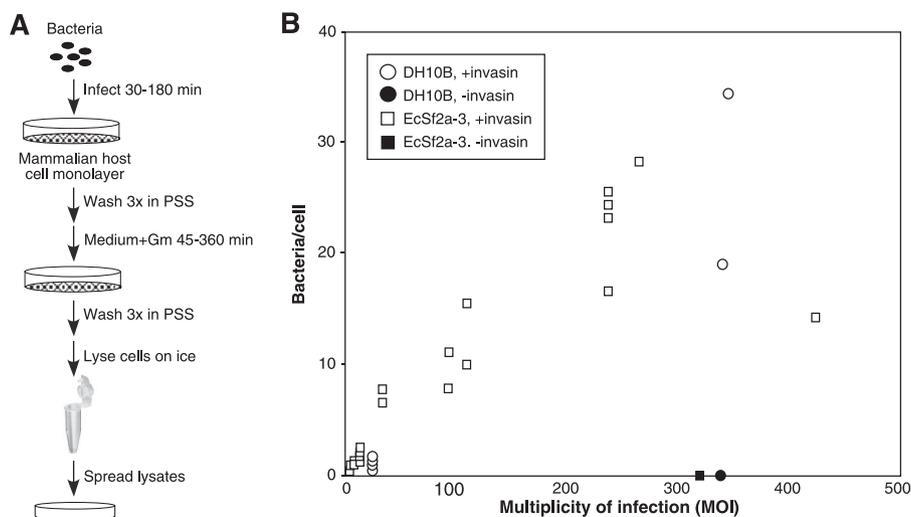
FIG. 1. Effect of invasin on infection of 3T3 cells. (A) Scheme of infection protocol. Briefly, confluent monolayers of mammalian cells were infected with bacteria at various MOIs (MOI of approximately 5 to 1,000, depending on the cell type) (see Materials and Methods for details) for 30 min to 3 h to allow the bacteria to invade the host cells. The infected cells were then incubated in medium containing Gm to kill extracellular bacteria. At the end of the procedure, the cells were washed and then lysed to release the intracellular bacteria, which were then counted by CFU. (B) Cellular entry into 3T3 cells of EcSf2a-3 and DH10B with and without invasin. Bacterial entry into 3T3 cells was plotted against the actual MOIs used (determined a posteriori) (see Materials and Methods for details).

ing the numbers of bacteria per cell without Cq for a given incubation time. For example, DH10B(pGB2Ω*inv*, RK2) infection of 143B cells resulted in 2.1×10^1 bacteria per cell, whereas EcSf2a-3(pGB2Ω*inv*, RK2) infection of 143B cells resulted in a comparable 1.3×10^1 bacteria per cell. Similarly, DH10B(pGB2Ω*inv*, pEGFP-N2) infection of 3T3 cells, with a 30-min incubation in Medium+Gm and a 45-min incubation in Medium+Gm+Cq, resulted in 1.0×10^0 bacteria per cell, while EcSf2a-3(pGB2Ω*inv*, RK2) infection under similar conditions resulted in 1.4×10^0 bacteria per cell.

However, only EcSf2a-3 was able to escape efficiently from vacuoles in 143B and 3T3 cells. In Fig. 2B (rows 1 and 2), for 143B cells and under similar experimental conditions, EcSf2a-3(pGB2Ω*inv*, RK2) resulted in a fivefold-higher percentage of Cq resistance, and, thus, of phagosomal escape, than DH10B(pGB2Ω*inv*, RK2). (The phagosomal escape percentage corresponds to the ratio of the numbers of bacteria recovered with and without Cq treatments.) The difference is more pronounced in 3T3 cells. The amount of escape for EcSf2a-3(pGB2Ω*inv*, RK2) infected into in 3T3 cells (Fig. 2C, black bars) increased with increasing incubation time in Medium+Gm, reaching 30% after 3 h. On the other hand, the amount of escape for pGB2Ω*inv*-encoding DH10B infected into 3T3 cells (Fig. 2C, white bars) was significantly lower, by 1 to 3 orders of magnitude, and decreased over time (DH10B escape decreases from 0.42% at 30 min to 0.03% at 180 min [Fig. 2B, rows 1 and 2], while EcSf2a-3 escape increases from 6.1% at 30 min to 30% at 180 min [rows 4 and 8]). The escape data are summarized in Fig. 2C. Note that in these experiments, plasmids pEGFP-N2 and RK2 were meant to serve only as antibiotic markers to select for cytosolic bacteria. These particular plasmids were used for their markers, and they had no effect on entry or escape. In the following experiments, we used 3T3 cells as the host cells for infection, as the percentage of pha-

gosome-escaping bacteria was much greater than that with infection of 143B cells.

Bacteria that gain access to the cytoplasm are able to conjugate. We designed an experimental protocol consisting of two rounds of bacterial infection into mammalian cells to look for cytosolic conjugation events (Fig. 3A). Briefly, the host cells were first infected with donor bacteria carrying a self-transmissible (conjugative) plasmid encoding Km^r, followed by infection with recipient Cm^r bacteria. In between the two rounds of infection, the cells were treated with Gm and Cq to kill extracellular and phagosomal bacteria. In this way, we ensured that donor and recipient bacteria did not encounter each other before they gained access to the cytoplasm, thus eliminating the possibility of conjugation between the two bacterial populations occurring outside of the cytoplasmic space. Since Gm does not penetrate the host cell membrane, we knew that we would be able to wash it away before the second round of infection. However, Cq is taken up by the mammalian cells, so we tested whether we could wash Cq out of the cells effectively to make sure that there was no residual Cq from the first round of bacterial infection that could interfere with the second round of infection (with optional Cq pretreatment) (Fig. 2A). We found that Cq can be washed out of 3T3 cells effectively and that subsequent bacterial infection and escape were not affected by multiple Cq treatments (Fig. 2B, last two rows). Even with Cq treatment, we observed a residual background level of escape for DH10B(pGB2Ω*inv*, RK2); however, these "escape" values decreased over time (Fig. 2B and C), indicating that we were detecting background events, perhaps because the bacteria were protected from the drugs in membrane ruffles and folds (18). In contrast, phagosomal escape reached 30% after 3 h for EcSf2a-3(pGB2Ω*inv*, RK2) (Fig. 2C). Vacuolar escape would presumably have continued to increase with longer incubation times; however, for reasons of expedi-

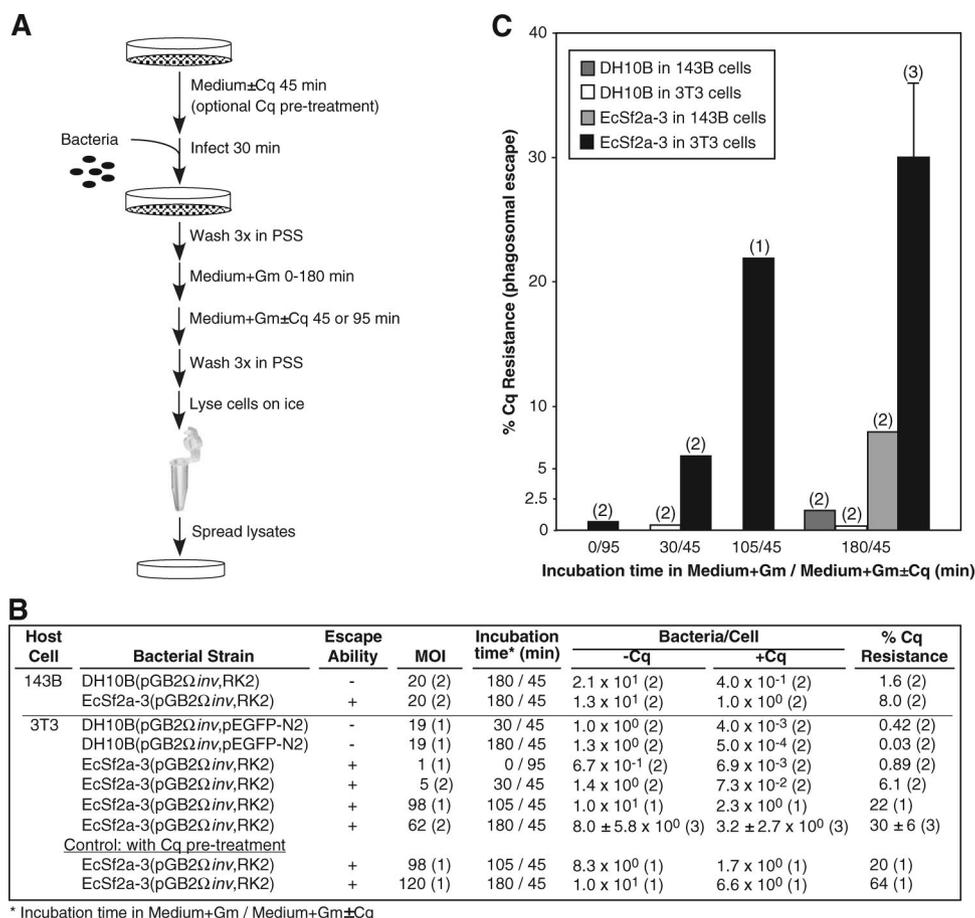| Host Cell	Bacterial Strain	Escape Ability	MOI	Incubation time* (min)	Bacteria/Cell −Cq	Bacteria/Cell +Cq	% Cq Resistance
143B	DH10B(pGB2Ωinv,RK2)	−	20 (2)	180 / 45	2.1×10^1 (2)	4.0×10^{-1} (2)	1.6 (2)
	EcSf2a-3(pGB2Ωinv,RK2)	+	20 (2)	180 / 45	1.3×10^1 (2)	1.0×10^0 (2)	8.0 (2)
3T3	DH10B(pGB2Ωinv,pEGFP-N2)	−	19 (1)	30 / 45	1.0×10^0 (2)	4.0×10^{-3} (2)	0.42 (2)
	DH10B(pGB2Ωinv,pEGFP-N2)	−	19 (1)	180 / 45	1.3×10^0 (2)	5.0×10^{-4} (2)	0.03 (2)
	EcSf2a-3(pGB2Ωinv,RK2)	+	1 (1)	0 / 95	6.7×10^{-1} (2)	6.9×10^{-3} (2)	0.89 (2)
	EcSf2a-3(pGB2Ωinv,RK2)	+	5 (2)	30 / 45	1.4×10^0 (2)	7.3×10^{-2} (2)	6.1 (2)
	EcSf2a-3(pGB2Ωinv,RK2)	+	98 (1)	105 / 45	1.0×10^1 (1)	2.3×10^0 (1)	22 (1)
	EcSf2a-3(pGB2Ωinv,RK2)	+	62 (2)	180 / 45	$8.0 \pm 5.8 \times 10^0$ (3)	$3.2 \pm 2.7 \times 10^0$ (3)	30 ± 6 (3)
	Control: with Cq pre-treatment						
	EcSf2a-3(pGB2Ωinv,RK2)	+	98 (1)	105 / 45	8.3×10^0 (1)	1.7×10^0 (1)	20 (1)
	EcSf2a-3(pGB2Ωinv,RK2)	+	120 (1)	180 / 45	1.0×10^1 (1)	6.6×10^0 (1)	64 (1)

* Incubation time in Medium+Gm / Medium+Gm±Cq

FIG. 2. Phagosomal escape. (A) Scheme of infection protocol. Host cell monolayers were infected with bacteria as described in the legend of Fig. 1A. Following treatment with Gm to kill extracellular bacteria, Cq was added to the medium in half of the wells to kill bacteria that were retained in the phagosomes. In one set of experiments, the host cell monolayers were pretreated with Cq prior to bacterial infection in order to verify that the drug can be washed out effectively so as not to interfere with subsequent bacterial infections. The infected cells were washed, lysed, and plated as described above to determine the percentage of bacteria that were able to escape from phagosomes. (B) Escape of invasin-encoding EcSf2a-3 and DH10B from vacuoles of 3T3 and 143B cells. Escape ability refers to whether or not the bacterial strain is capable of lysing the phagosomal membrane to gain access to the host cell cytoplasm. MOIs were determined a posteriori (see Materials and Methods for details) and were determined once for a set of experiments run at the same time (i.e., on the same day); numbers of experiments are indicated in parentheses (e.g., MOIs with $n = 2$ indicate that the data were gathered from experiments run on two different days). Incubation times are given for treatment in Medium+Gm and Medium+Gm+Cq. The numbers of bacteria per cell were calculated as described above (numbers in parentheses indicate the total numbers of independent experiments performed, with the standard errors of the means [SEM]). The percentage of phagosomal escape is given by %Cq resistance and was calculated as follows: (number of bacteria recovered with Cq treatment/number of bacteria recovered in the absence of Cq treatment) × 100. The percentages of Cq resistance were calculated for each experiment and then averaged for the reported results. Note that in these experiments, all bacterial strains contain the invasin plasmid pGB2Ωinv. In addition, the bacteria also contained a second plasmid, encoding an antibiotic marker to select for cytosolic bacteria. For these experiments, RK2 or pEGFP-N2 was used. (C) Graphical presentation of escape data for experiments in which host cells were not pretreated with Cq prior to bacterial infection (first eight rows of data in B). Numbers of experiments are indicated in parentheses; error bars indicate SEM.

ency in the double-infection experiments, we incubated cells in Medium+Gm for 2 to 3 h prior to Cq treatment (Fig. 3). We detected conjugation events by assaying for self-transfer of the donor Km^r plasmid (RK2) into Cm^r (pACYC184-containing) recipient bacteria within the cytoplasm of the host cells to give easily selectable, doubly resistant, Km^r Cm^r transconjugant bacteria.

Both donor and recipient bacteria carried the non-self-transmissible, Sp-resistant invasin plasmid pGB2Ωinv to enhance cellular entry. In addition, donor bacteria carried the broad-host-range, self-transmissible plasmid RK2, which confers resistance to Km and sensitivity to Sp and Cm. Recipient bacteria carried the non-self-transmissible plasmid pACYC184, which confers resistance to Cm and sensitivity to Sp and Km. When donor bacteria [e.g., EcSf2a-3(pGB2Ωinv, RK2)] encounter recipient bacteria [e.g., EcSf2a-3(pGB2Ωinv, pACYC184)], plasmid RK2 is transferred into the recipients. The resulting transconjugants [e.g., in this case, EcSf2a-3(pGB2Ωinv, RK2, pACYC184)] containing plasmid-borne genes conferring resistance to Km and to Cm are able to grow in the presence of both antibiotics, whereas donor bacteria can grow on medium containing Km or Km and Sp, and recipient bacteria can grow on medium containing Cm or Cm and Sp. Neither donors nor recipients can grow on medium containing both Km and Cm.
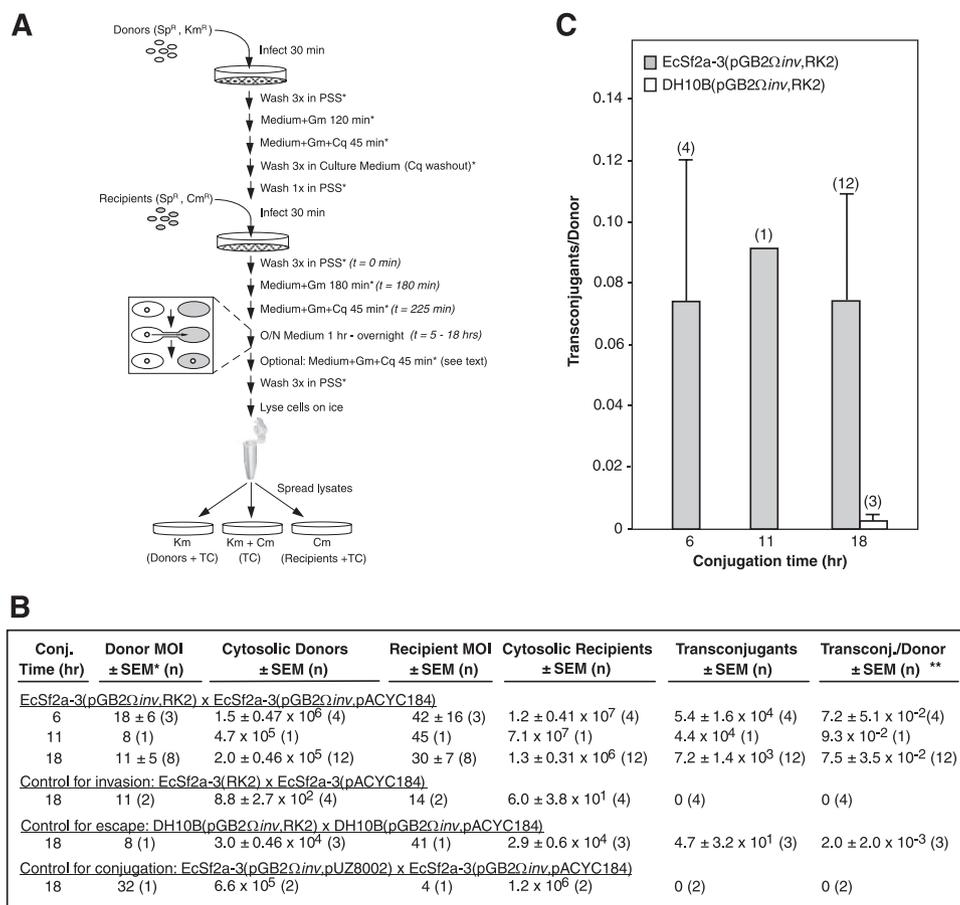
FIG. 3. Intracellular conjugation. (A) Scheme of the intracellular conjugation protocol. Host cell monolayers were first infected with donor bacteria. The donors were allowed to invade for 30 min before treatment with Gm to eliminate extracellular bacteria, followed by treatment with Gm and Cq to eliminate bacteria remaining in the phagosomes. The monolayers were then infected with recipient bacteria. After the second treatment with Gm and Cq, the cells were incubated for various lengths of time (1 h to overnight) to allow conjugation to occur between the cytosolic donors and recipients. In some cases, the monolayers were treated with Gm and Cq a third time prior to lysis and spreading onto plates with Km plus Sp to determine the number of cytosolic donors, onto plates with Cm plus Sp to determine the number of cytosolic recipients, and onto plates with Km plus Cm to determine the number of transconjugants (TC). Asterisks indicate steps in the protocol where media and/or washes were sampled to check for the presence and amount of relevant bacteria. (B) Tabular presentation of conjugation data. Matings are depicted as donor × recipient. Conjugation time represents the length of time between the second Cq treatment and the third Cq treatment or lysis. MOIs and numbers of experiments are as described in the legend of Fig. 2. The numbers of transconjugants per donor were calculated for each experiment and then averaged for the reported results. (C) Graphical presentation of conjugation data, represented as transconjugants per donor for EcSf2a-3(pGB2Ωinv, RK2), compared to data for the DH10B(pGB2Ωinv, RK2) control for phagocytic escape. Numbers of experiments are indicated in parentheses; error bars indicate SEM.

At the end of the experiment, the 3T3 cells were lysed and spread onto plates with appropriate drug selection to determine the numbers of donor, recipient, and transconjugant bacteria. Samples of media and washes were taken (indicated by asterisks in Fig. 3A) to check for the presence and amounts of the various bacterial species; these "intermediate" samples confirmed our expectations that the washing procedures had their intended effects of removing extracellular and vacuolar bacteria (data not shown). Due to the length of the experimental protocol, it was most convenient to assay for conjugation about 18 h after infection of cells by the recipient bacteria (i.e., overnight), but some assays were performed at earlier time points (1, 6, and 11 h).

We previously confirmed that the strains used here are competent for conjugation in vitro before proceeding with conjugation inside cultured cells (data not shown). We were able to demonstrate here that conjugation also occurs between bacteria in the cytosol (Fig. 3). The average numbers of transconjugants per donor were initially low (7×10^{-4} transconjugants per donor after 1 h) (not shown) and increased to 7×10^{-2} to 9×10^{-2} transconjugants per donor at the remaining time points examined (6, 11, and 18 h) (Fig. 3B and C). We note that the numbers of EcSf2a-3 donors and recipients present in the cytosol decreased by an order of magnitude between 6 and 18 h (Fig. 3B). This decline was not the result of host cell death (data not shown); rather, it was probably due to the fact that EcSf2a-3 is an $aroD$ mutant. Auxotrophic $aroD$ mutants are unable to survive for extended periods within the mammalian cytosol because they require $para$-aminobenzoic acid for purine nucleotide biosynthesis, and $para$-aminobenzoic acid is not

present in eukaryotic cells (66), nor is it present in the culture medium. However, due to stores of purine nucleotides and/or metabolites in both the bacterial cells and the mouse cell cytoplasm, the bacteria were able to survive and conjugate for a short period (at least the first 6 h after infection) until those stores of nucleotides and metabolites were depleted. Our results imply that conjugation continues to occur even when nucleotides or metabolites are limiting for vegetative replication, since the numbers of transconjugants per donor remained high (7×10^{-2} transconjugants) after 18 h, whereas the numbers of donors and recipients decreased dramatically (by 2-fold and 50-fold, respectively). No transconjugants were obtained in control experiments where 3T3 cells were infected with EcSf2a-3 bacteria lacking the invasin plasmid and thus deficient for cellular entry (Fig. 3B). Also, physical analyses of the plasmid DNA from several transconjugants showed that the plasmids in the transconjugants had the same structures as the plasmids in the respective donors and recipients (data not shown).

DH10B bacteria carrying the invasin plasmid were able to enter cells efficiently, but we expected intracellular DH10B to remain trapped in vacuoles. However, Cq treatment allowed us to detect a low level of vacuolar escape of DH10B bacteria. These bacteria were also competent for conjugation (Fig. 3B and C). These presumed "escapees" may reflect the background level of bacterial survival in phagosomes following treatment with Cq. In our protocol, we omitted Cq from the medium during incubations greater than 1 h because prolonged treatment with Cq is toxic to cells (12). It is formally possible that some DH10B bacteria may have escaped the two rounds of Cq treatment and conjugated inside phagosomes, thus artificially inflating the cytoplasmic conjugation values. To address this concern, we performed experiments in which Cq was added a third time, just prior to lysis, to eliminate any vacuolar transconjugants. We found no significant difference in conjugation under the two conditions (data not shown), implying that conjugation was occurring in the cytoplasm and not in vacuoles. Furthermore, the average number of transconjugants per donor for EcSf2a-3 (escape-competent bacteria) was more than 30-fold higher than that for DH10B (escape-incompetent bacteria) (Fig. 3B) despite having similar conjugation rates in vitro (data not shown).

Are the Km^r Cm^r bacteria the result of conjugation? Some bacteria exhibit a natural competence for transformation by naked DNA (34). In our experiments, we observed that there are bacteria that do not survive in the cytoplasm. Theoretically, the dead bacteria could release DNA, and some of the Km^r Cm^r bacteria could be transformants as opposed to bona fide transconjugants. Natural competence for transformation has not been observed for *Escherichia coli* or *Shigella flexneri* in the laboratory. Moreover, we proved directly that we observed conjugation, and not transformation, in our experiments by using a strain of EcSf2a-3 carrying the *oriT*-defective plasmid pUZ8002. These bacteria are competent for both invasion and escape but not for the efficient self-transfer of pUZ8002 by conjugation. Plasmid pUZ8, from which pUZ8002 is derived, is almost identical and is highly homologous to RK2; it is thus a suitable control. In addition, both pUZ8 and RK2 conjugate by the IncPα conjugation system, and their origins of transfer are identical. Even though the cellular infectivity of donor EcSf2a-3(pGB2Ω*inv*, pUZ8002) was similar to that of EcSf2a-3(pGB2Ω*inv*, RK2), not a single Km^r Cm^r colony was observed at 18 h when using the conjugation-defective strain (Fig. 3B). This result implies that in the cytoplasm, transformation with these bacteria by naked DNA is a very rare event, if it occurs at all. We conclude that all or nearly all of the Km^r Cm^r bacteria that we observed arose by conjugation.

As the EcSf2a-3(pGB2Ω*inv*, RK2) strain can conjugate in vitro (data not shown), another possibility is that the conjugation that we observed occurred after the lysis of host cells, i.e., either in the tube before plating or on the plates before the lysates were absorbed into the agar. To test this possibility, we infected one plate of 3T3 cells with donors and another plate with recipients (followed by Gm and Cq treatment), lysed the cells on the two plates on ice, and then mixed the lysates and looked for transconjugants (Fig. 4A). This experiment yielded a postlysis average background conjugation rate of approximately 10^{-4} transconjugants per donor (Fig. 4B). Thus, any rates of conjugation above this value should be indicative of authentic cytosolic conjugation. The conjugation value obtained for EcSf2a-3(pGB2Ω*inv*, RK2), approximately 7×10^{-2} to 9×10^{-2} transconjugants per donor, is greater than 2 orders of magnitude higher, indicating a high rate of genuine cytosolic conjugation events.

DISCUSSION

While it has been shown that *Salmonella enterica* serovar Typhimurium can conjugate in the vacuolar compartments within host cells (20), to our knowledge, it has never been demonstrated that conjugation can take place in the cytosol. Using an *Escherichia coli-Shigella flexneri* hybrid bacterium expressing *Yersinia pseudotuberculosis* invasin, we have shown here that bacteria can conjugate in the cytosol of mammalian cells. Our results have implications for antibiotic therapy, for the evolution of intracellular bacteria, and for new methods to engineer mammalian cells.

We modified two strains of *E. coli* (EcSf2a-3 and DH10B) such that they were taken up rapidly by mammalian cells. The avirulent vaccine strain EcSf2a-3 is an *E. coli* strain that contains some virulence genes (both chromosomal and plasmid borne) from *S. flexneri* (2). DH10B is a strain commonly used for molecular cloning (22). We introduced a plasmid encoding the invasin protein from *Y. pseudotuberculosis* into both EcSf2a-3 and DH10B to enable them to enter cells efficiently. Once inside, only the EcSf2a-3 strains escaped from phagocytic vacuoles to access the cytoplasm, where we observed high levels of conjugation. In contrast, the DH10B strains remained mostly in the endosomal compartment, and we were able to detect only a low level of conjugation. Our results show that bacteria can exchange an antibiotic resistance plasmid in the cytosol of infected cells.

The extent to which cytoplasmic conjugation is a general phenomenon remains to be established, but it may well extend to other intracellular bacteria and host cells. The levels of cytoplasmic conjugation could be higher in cells that are the natural targets for pathogens. The invasion of those cells should be common, e.g., epithelial cells of the colonic mucosa (25). Although we did not look for cytosolic conjugation between wild-type shigellae, it is possible that virulence factors in

Donor MOI ± SEM* (n)	Cytosolic Donors ± SEM (n)	Recipient MOI ± SEM (n)	Cytosolic Recipients ± SEM (n)	Transconjugants ± SEM (n)	Transconj./Donor ± SEM (n)**
EcSf2a-3(pGB2Ωinv,RK2) x EcSf2a-3(pGB2Ωinv,pACYC184)					
16 (2)	$1.0 \pm 0.29 \times 10^5$ (3)	38 (2)	$5.4 \pm 0.72 \times 10^5$ (3)	$1.2 \pm 0.6 \times 10^{-1}$ (3)	$1.0 \pm 0.8 \times 10^{-4}$ (3)
Control for conjugation: EcSf2a-3(pGB2Ωinv,pUZ8002) x EcSf2a-3(pGB2Ωinv,pACYC184)					
20 (2)	$3.5 \pm 0.24 \times 10^5$ (3)	38 (2)	$4.5 \pm 0.48 \times 10^5$ (3)	0 (3)	0 (3)

* Standard error of the mean. ** Average of values determined in individual experiments.
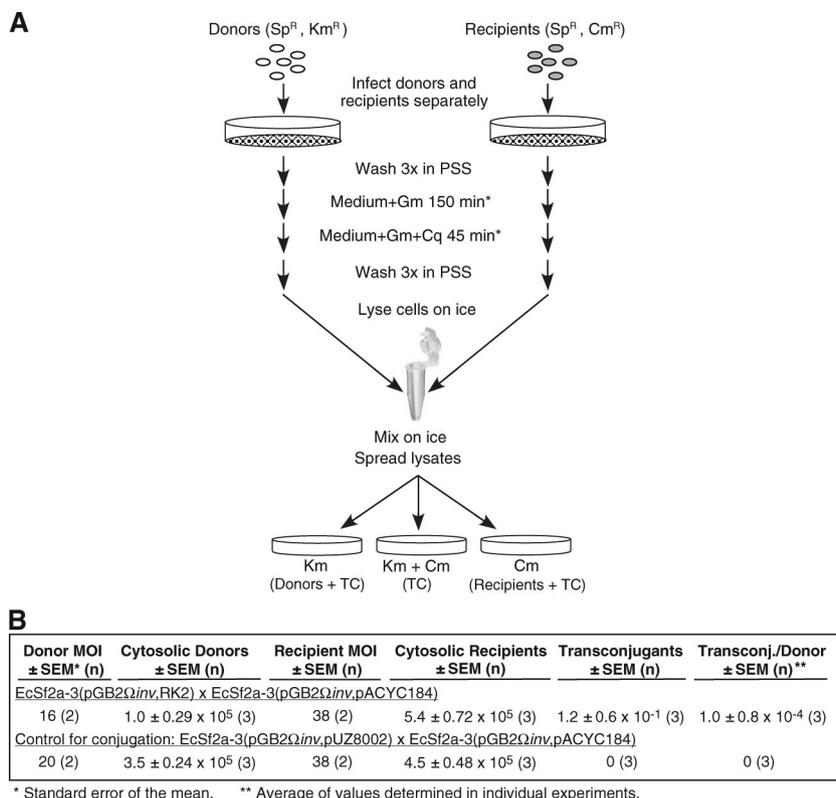
FIG. 4. Assay for postlysis conjugation. (A) Scheme of infection protocol. Donor and recipient bacteria were infected into separate monolayers and allowed to invade the host cells. The infected cells were then treated with Gm and Gm then Cq, followed by lysis, as was done earlier. The donor-infected lysates were mixed on ice with the recipient-infected lysates and then plated with selection for donors, recipients, and transconjugants in order to determine the background conjugation rate during the processing steps after cell lysis. Asterisks are as described in the legend of Fig. 3. (B) Tabular presentation of the postlysis conjugation data. Matings are depicted as donor × recipient. MOI and numbers of experiments are as shown in Fig. 2. The numbers of transconjugants per donor were calculated for each experiment and then averaged for the reported results.

wild-type *S. flexneri* that are not expressed in the avirulent strain EcSf2a-3 used here may result in more frequent and faster vacuolar escape. Thus, conjugation between wild-type *S. flexneri* in the cytoplasm may be more frequent than in EcSf2a-3. On the other hand, *virG* allows *S. flexneri* to move quickly from one cell to another, reducing the amount of time spent in the original cell. This ability might lower the rate of cytosolic conjugation between *S. flexneri* strains compared to that of the "trapped" EcSf2a-3 *virG* mutant. Nevertheless, there is good reason to believe that conjugation can occur with natural intracellular pathogens. We note that obligate intracellular pathogens such as the *Rickettsia* spp. (51) harbor conjugative plasmids and transposons (55). Also, F-like plasmids are able to avoid natural barriers to horizontal gene transfer in diverse ecological niches (48).

The use of antibiotics has become commonplace. Disinfectants are present in the food that we consume, in household cleaning products, and in items for personal hygiene such as bactericidal soaps. The overprescription of antibiotics is rampant (61), and antibiotic use in hospital settings is pervasive. The ability of bacteria to develop and share resistance determinants quickly and to become multiply resistant by exchanging genes is a serious problem. Nosocomial infections with multiply resistant bacteria are becoming a great concern and present unique challenges (4, 38). In addition, the administra-

tion of antibiotics at subtherapeutic levels to promote animal growth (30) and as a prophylactic measure against infection (a common practice in animal husbandry in North America) (6) is probably futile and may even exacerbate the severity of infections caused by pathogenic intracellular bacteria (37).

While antibiotics are necessary to control and prevent the spread of infection, there are few new antibiotics today. As more was learned about how different pathogens work, it was discovered that some bacteria are able to enter the cytoplasm of host cells. Unfortunately, the cellular cytoplasmic environment is largely protected from antibiotics (19, 35, 46, 53) because the drugs either are unable to penetrate the plasma membrane of cells, are pumped out, or are inactivated intracellularly (e.g., in lysosomes). Therefore, intracellular pathogenic bacteria have an advantage over free bacteria because they can evade the effects of antibiotics that are unable to cross the plasma membrane. To quote Maurin and Raoult (45), "the intracellular location of several bacteria and fungi has been known for decades as a critical point to explain failure of antibiotic treatment to eradicate these pathogens from host cells." Because conjugation can mobilize any gene, our results infer that a gene that encodes an antibiotic resistance or virulence factor can be transferred in the cytosol. Many antibiotic resistance and virulence determinants are plasmid encoded (36), which contributes to their spread and evolution via horizontal

gene transfer. Furthermore, antibiotics that are unable to be effective inside cells provide protection against surface pathogens but not against intracellular pathogens. Moreover, as plasmids can be received and transmitted by "dead" bacteria during antibiotic treatment (27), developing antibiotics that merely target the biofilm or intracellular space is insufficient to halt the spread of resistance elements. Thus, for the more effective treatment of intracellular bacterial infections and to prevent gene exchange within the cytoplasm, it would be of value to develop antibiotics that act intracellularly and target the conjugation machinery itself, underscoring the need to understand the conjugation process as it occurs within host cells. We currently lack antibiotics that can penetrate cells effectively and act on intracellular organisms. Furthermore, bacteria that are killed by antibiotics can still conjugate with other bacteria (27). Further study of gene transfer in this niche will help us understand the conditions under which antibiotic resistance and virulence genes are transferred. Additional research will also assist in the development of better antimicrobial agents and new strategies for treatment.

Bacterial conjugation inside eukaryotic cells may provide an effective method for introducing foreign DNA into mammalian cells. It may be possible to use intracytoplasmic bacteria to deliver large pieces of DNA to the nucleus or to mitochondria/chloroplasts via conjugation. In this regard, we note that mitochondria have been observed in close proximity to *S. flexneri* within the cytosol of infected cells, and in many cases, membrane-to-membrane contact was observed (13, 43). Conjugative plasmids provide donor bacteria with all the protein factors for the mobilization and transfer of DNA from donor to recipient. As a consequence, conjugation is not restricted to prokaryotic recipients. Some donor bacteria can conjugate with other cell types (40). Conjugation is already a mechanism for the interkingdom exchange of genetic information. Interkingdom conjugation by bacteria has been demonstrated not only in plants (7, 40) but also in yeast (28), in mammalian cells (68), and in mitochondria isolated from mouse cells (69). DNA has been introduced successfully into fungal and plant mitochondria (8, 17, 63). However, there are currently no practical methods for introducing exogenous DNA into mammalian mitochondria in a stable and heritable manner. The design of an avirulent bacterium that is capable of conjugation in the cytosol opens up the possibility of transferring DNA across intracellular membranes of organelles such as mitochondria, chloroplasts, and nuclei.

ACKNOWLEDGMENTS

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