Genetic and molecular analysis of the virulence plasmid of *Salmonella enterica* serovar Typhimurium

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Genetic and molecular analysis of the virulence plasmid of *Salmonella enterica* serovar Typhimurium

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“La paciencia todo lo alcanza”

Teresa de Jesús

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CONTENTS

ABBREVIATIONS

CI	Competitive index
DT	Definitive phage type
ET	Electrophoretic type
LD_{50}	Lethal dose 50
LT	Lilleengen type
M cell	Microfold cell
MLEE	Multilocus enzyme electrophoresis
RCR	Rolling circle replicating
SCFA	Short-chain fatty acid
SDS	Sodium dodecyl sulfate
SPI	*Salmonella* pathogenicity island
ST	Sequence type
T3SS	Type III secretion system
T4SS	Type IV secretion system
VR	Virulence-resistance

RESUMEN

Salmonella enterica serovar Typhimurium posee un plásmido de gran tamaño (93 Kb) llamado plásmido de virulencia o pSLT. Dicho plásmido contiene el locus *spv*, esencial para la infección sistémica en roedores. El plásmido fue presumiblemente adquirido por el hospedador ancestral de *Salmonella* mediante conjugación. En la mayoría de las estirpes del serovar Typhimurium, el plásmido pSLT contiene un operón *tra* completo. En esta tesis se han realizado diversos análisis estructurales y funcionales en el plásmido pSLT, habitualmente empleando estrategias genéticas (sobre todo, uso de mutantes).

En condiciones de laboratorio, la adición de desoxicolato sódico a los cultivos permite la detección de aislados curados del plásmido. Ello sugiere que la estabilidad de pSLT puede hallarse comprometida en vivo, ya que las sales biliares alcanzan concentrationes notables en el intestino delgado y especialmente en el duodeno. La síntesis de los pili conjugativos codificados por pSLT no causa sensibilidad a bilis, a diferencia de lo que ocurre con los pili codificados por el plásmido F. Es posible que el paso de *Salmonella* por el tracto hepatobiliar a lo largo de millones de años haya seleccionado variantes del plásmido de virulencia resistentes a bilis.

La frecuencia de curación de pSLT en presencia de desoxicolato sódico aumenta en un mutante carente del módulo de adicción CcdAB. Ello sugiere que dicho módulo es funcional en *Salmonella*.

La posibilidad de que el plásmido de virulencia sufra curación en el duodeno plantea la posibilidad de que la conjugación tenga un alto valor selectivo durante la infección de animales. Dado que las células curadas del plásmido de virulencia tienen menor capacidad de infección sistémica, recuperar el plásmido pSLT en el propio intestino podría tener valor adaptativo.

La transferencia del plásmido de virulencia durante la infección del ratón se detecta con relativa facilidad, ya que las frecuencias de conjugación son sorprendentemente altas. Sustancias típicas del duodeno (bilis) y del intestino grueso (propionato) inhiben la frecuencia de transmisión conjugativa de pSLT. Perece probable que la conjugación dentro del ratón tenga lugar en el íleon. Esta hipótesis es apoyada por la detección de transconjugantes a alta frecuencia en asas de íleon de ratón.

Un factor clave en la regulación de la transferencia conjugativa de pSLT es la actividad del sistema FinOP de inhibición de la fertilidad. Un ensayo de análisis transcriptómico mostró que el ARN FinP tenía dianas en el cromosoma. Una de ellas es el gen biosintético *cysD*. En presencia de RNA FinP, la cantidad de transcrito *cysDNC* aumenta. También se observa un aumento a nivel de proteína. La deleción del gen *cysD* aumenta la frecuencia de conjugación un orden de magnitud, proporcionando un indicio de que la biosíntesis de cisteína afecta a la conjugación. Ya que la proteína CysD está implicada en la ruta biosintética de la cisteína a partir de sulfato, se estudió si el fenotipo conjugativo de la deleción era debido a una segunda función de CysD o a su implicación en la biosíntesis de L-cisteína. La interrupción de la ruta en otro paso indicó que la biosíntesis de cisteína estaba implicada en la transferencia del plásmido. Además, los productos finales de la ruta (sulfuro y cisteína) producen una disminución en la frecuencia de conjugación. Por tanto, FinP inhibe la transferencia del plásmido de virulencia a través de dos vías: i) como ARN antisentido de *traJ*; e ii) aumentando los niveles de L-cisteína a través de dianas cromosómicas en *trans*, y específicamente aumentando la expresión del operón *cysDNC*.

Finalmente, se ha estudiado la posible adaptación de los plásmidos de virulencia a su hospedador. Para ello se calcularon las frecuencias de conjugación de tres estirpes diferentes: LT2, ATCC 14028 y SL1344. Cada estirpe mostró tener una frecuencia específica de conjugación, que dependía únicamente del propio plásmido. En la estirpe SL1344 la frecuencia de conjugación era más baja, y la causa principal parece hallarse en una mutación en el gen *traG*. Sin embargo, los tres plásmidos son totalmente intercambiables para la virulencia. Estos resultados, junto con la abundancia de deleciones en los operones *tra* de otros serotipos (y en menor medida en aislados naturales del serovar Typhimurium) sugieren que los genes *spv* están sometidos a selección, mientras que los genes *tra* son más o menos neutros. Si esta interpretación es correcta, parece plausible que los plásmidos de virulencia de *Salmonella* evolucionen hacia formas no autotransmisibles.

INTRODUCTION

1. The genus *Salmonella*

Bacteria of the genus *Salmonella* are Gram-negative nonspore-forming bacilli that belong to the *Enterobacteriaceae* family. Salmonellae are motile (except *Salmonella enterica* serovar Pullorum and Gallinarum) and express peritrichous flagella. The genus *Salmonella* is phylogenetically close to the genera *Escherichia*, *Shigella* and *Citrobacter*. The general organization of the *Salmonella* genome is similar to that of *Escherichia coli* (Groisman & Ochman, 1994; Sanderson *et al.*, 1995). However, *Salmonella* harbors specific A+T-rich regions acquired by lateral transfer that are not found in related genera. Some such regions are involved in pathogenesis and are thus called *Salmonella* pathogenicity islands (SPI).

The genus *Salmonella* consists of two species: *S. enterica* and *S. bongori* (Tindall *et al.*, 2005). Both species have the capacity to invade host cells due to the possession of pathogenicity island 1 (SPI-1), but only *S. enterica* has the capacity for systemic spread, due to the possession of another pathogenicity island known as SPI-2 (Groisman & Ochman, 1997; Hensel, 2000). *S. enterica* is divided in six subspecies (Tindall *et al.*, 2005): *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI). Another group, designated subspecies VII, was identified by multilocus enzyme electrophoresis (MLEE) analysis, but contained only four isolates initially allocated to subspecies IV on the basis of biochemical characteristics (Boyd *et al.*, 1996). At the sequence level it is as distinct as some other subspecies, but there is no phenotypic description or taxonomic name yet.

Every *Salmonella* subspecies is classified in serovars. *Salmonella* serovars are distinguished by antisera to two highly variable surface antigens, the O antigen and the H antigen, reflecting variation in the exposed part of lipopolysaccharide and in flagellin, respectively (Grimont, 2007; McQuiston *et al.*, 2004). There are more than 2500 serovars (Grimont, 2007). Most serovars were initially given Latin binomial species names, which have been retained as serovar names (Brenner *et al.*, 2000; Le Minor, 1987). For example, the original *S. typhimurium* is now referred to as *S. enterica* serovar Typhimurium (or *S.* Typhimurium).

Salmonella bongori and *Salmonella enterica* subspecies II, IIIa, IIIb, IV and VI are commonly isolated from cold-blood vertebrates or from the environment whereas subspecies I, with more than 1300 serovars, is generally isolated from mammals or birds (Baumler *et al.*, 1998; Boyd *et al.*, 1996). In subspecies I there is

a considerable overlap between human and farm-animal serovars (Alcaine *et al.*, 2006; Foley *et al.*, 2008). Other serovars are host specific or confined to a small number of hosts. For instance, the serovars Typhi and Paratyphi A cause typhoid fever only in humans and higher primates (Edsall *et al.*, 1960).

When non-typhoid *Salmonella* passes from domestic animals to the food chain, humans can become an accidental host and suffer from a short term enterocolitis named salmonellosis. Seventy percent of salmonellosis cases are caused by only 12 subspecies-I serovars (Anjum *et al.*, 2005). According to the *Salmonella* Annual Summary 2006, *S.* Typhimurium was the most common serotype of foodborne illnesses, causing 16.9% of all human salmonellosis. *S.* Enteritidis was ranked number two, causing 16.3% of all human cases of salmonellosis.

1.1 Diversity of *S.* Typhimurium

S. enterica serovar Typhimurium has been traditionally considered the prototypical broad-host-range serotype since it is frequently associated with disease in numerous species, including humans, livestock, domestic fowl, rodents, and birds. However, some serovar Typhimurium variants have a very narrow host range, while others are able to infect and persist in multiple species. Therefore, serovar Typhimurium may be described as a collection of variants that vary significantly in their host range and their degree of host adaptation (Rabsch *et al.*, 2002).

In order to distinguish serovar Typhimurium variants, a number of techniques to monitor diversity have been developed.

Multilocus enzyme electrophoresis analysis of 300 isolates using 20 enzymes found 23 electrophoretic types (ETs) (Beltran *et al.*, 1991). The majority of isolates belong to ET Tm1, and all *S.* Typhimurium ETs were found to be grouped together with a number of other serovars in a group called the Typhimurium clonal complex (Beltran *et al.*, 1991). Recent multilocus sequence typing has confirmed this picture.

The *S.* Typhimurium isolates publicly available in the *Salmonella* MLST database have been grouped into 12 sequence types (STs). Nine STs belong to a single clonal complex with ST19 as the founder (Lan *et al.*, 2009).

Phage typing distinguishes serovar Typhimurium variants based on their susceptibility to a set of phages. This method provides a rapid, accurate and inexpensive method to characterize *Salmonella* strains during epidemiological studies (Anderson *et al.*, 1978). Several phage typing systems have been described. In 1948, Lilleengen distinguished 24 Lilleengen types (LT) among serotype Typhimurium isolates (Lilleengen, 1948). However, since 1996, the phage typing system originally described by Felix and Callow (Felix, 1943) is used. An extended version of this typing system (Anderson, 1977) distinguishes more than 300 definitive phage types (DT). DTs can be further differentiated by biotyping, a method based on fermentative characteristics (Anderson *et al.*, 1978; Duguid, 1975; Edwards, 1936).

Phage typing illustrates the diversity of Typhimurium and provides important epidemiological information. However, the relationships between phage types cannot be inferred from the phage sensitivity patterns because phage types can change with rather few genetic modifications, often involving a gain or loss of mobile elements (Mmolawa *et al.*, 2002).

LT2, ATCC 14028 and SL1344 are three *S.* Typhimurium strains commonly used in the laboratory. Since its original isolation in the 1940s by Lilleengen, *S.* Typhimurium LT2 has been widely used in genetic studies (Lilleengen, 1948; Sanderson, 1987; Zinder, 1952). The descendants of the original LT2 isolate are most accurately described as a set of clades, most of which are highly attenuated for virulence in susceptible mice due to a *rpoS* mutation (Bearson *et al.*, 1996; Sanderson, 1987; Swords *et al.*, 1997). This fact has led many investigators to use other *S.* Typhimurium virulent strains, such as SL1344, from the B.A.D. Stocker collection, or ATCC 14028, which is a descendant of CDC 60-6516 (a strain isolated in 1960 from pools of hearts and livers of 4-week-old chickens by P. Fields), to examine the genetic basis of bacterial pathogenesis (Fields *et al.*, 1986; Gulig & Curtiss, 1987; Harrington & Hormaeche, 1986).

The main chromosomal differences among these three representative strains are the presence or absence of several prophages. The three strains are lysogenic for two temperate bacteriophages, Gifsy-1 and Gifsy-2 (Figueroa-Bossi *et al.*, 2001). ATCC 14028 and SL1344 also carry the St64B prophage (Figueroa-Bossi & Bossi, 2004). Furthermore, every strain carries specific prophages. Fels-1 and Fels-2 are present only in LT2 although there are remnants of Fels-2 genes at the

corresponding position in the ATCC 14028 genome (Figueroa-Bossi *et al.*, 2001). Phage Gifsy-3 is only present in the strain ATCC 14028, while SL1344 carries the phage SopEΦ (Figueroa-Bossi *et al.*, 2001). The latter prophage has been associated with a group of strains responsible for epidemic outbreaks in the United Kingdom and former East Germany in the 1970s and 1980s (Mirold *et al.*, 1999; Prager *et al.*, 2000). Strain SL1344 has additional specific traits, such as histidine auxotrophy, streptomycin resistance and incapability of forming biofilms as a consequence of mutations in *hisG*, *rpsL*, STM1987 and *mlr*A genes (Garcia *et al.*, 2004; Hoiseth & Stocker, 1981). These observations suggest that these three strains have further evolved after their isolation from nature.

2. Virulence of *S. enterica* serovar Typhimurium

S. Typhimurium causes gastroenteritis in humans and typhoid-like fever in mice. For this reason, *S.* Typhimurium is commonly used as a model for the systemic disease promoted by serovar Typhi in humans. In contrast, use of mice for the study of gastroenteritis presents the limitation that these animals do not develop exudative inflammation in the intestinal mucosa during *S.* Typhimurium infection. However, this limitation can be overcome by pretreatment of mice with streptomycin (Hapfelmeier & Hardt, 2005). Calf is another suitable animal model for the study of gastroenteritis because infection with *S.* Typhimurium results in localized enteric disease with clinical and pathological features reminiscent of the enteric disease in humans (Zhang *et al.*, 2003).

The BALB/c mouse is especially sensitive to intracellular pathogens due to lack of the macrophage-associated protein Nramp1. This protein is necessary to control intracellular replication of microbes such as *Salmonella*, *Mycobacterium* and *Leishmania* (Bellamy, 1999; Govoni & Gros, 1998; Gruenheid *et al.*, 1997).

The classical method to identify an attenuating mutation using mice is the calculation of the lethal dose 50 (LD_{50}), compared with the LD_{50} for the wild-type strain (Reed, 1935). Another procedure is infection of a mouse with two strains, to obtain a sensitive measure of virulence attenuation known as the competitive index (CI). The CI is defined as the ratio between the mutant strain and the wild type in the output divided by the ratio of the two strains in the input (Freter *et al.*, 1981; Taylor *et al.*, 1987). This method is an alternative to LD_{50}, and has two important

advantages: (i) less animals are needed to obtain statistically significant results; (ii) because both strains are tested simultaneously in the same animal, the variability inherent to the use of different animals is avoided. A refinement of this method, involving mixed infections of single and double mutant strains, allows the study of virulence gene interactions *in vivo* (Beuzon & Holden, 2001).

S. Typhimurium is usually transmitted to the host by ingestion of contaminated food or water. The digestive system typically combats potentially pathogenic microbes through the production of several bactericidal agents along the tract.

The first antibacterial agents produced in the gastrointestinal tract are gastric secretions and hydrochloric acid. Both lower the pH of the stomach to approximately 3.0. This acidic environment destroys the majority of bacteria that enter the stomach (McGowan *et al.*, 1996; Tennant *et al.*, 2008). However, *S.* Typhimurium, which prefers to live and grow at a pH near neutrality, responds to acidic challenges through a complex adaptive system called the acid tolerance response, in which adaptation to mild (pH 5.8) or moderate (pH 4.4) acid conditions enables the cell to endure periods of severe acid stress (pH 3) (Foster & Hall, 1990; Lee *et al.*, 1994; Lee *et al.*, 1995).

Another bactericidal agent found in the digestive system is bile, a complex fluid containing bile salts, cholesterol, bilirubin, and other organic molecules (Hofmann, 1998). Primary bile acids (or bile salts) are synthesized in the liver from cholesterol (Hofmann, 1999; Okoli *et al.*, 2007). Further metabolism results in the formation of secondary bile salts. Bile is concentrated and stored in the gallbladder during fasting and released through the bile duct into the duodenum during food passage (Ridlon *et al.*, 2006). The bile salts released into the intestine are further metabolized into secondary and tertiary bile salts by intestinal bacteria (Hay & Carey, 1990; Monte *et al.*, 2009; Ridlon *et al.*, 2006). The most abundant bile salts in humans are cholate and deoxycholate.

Bile salts have at least two distinct antibacterial activities, as detergents that disrupt the cell envelope (Gunn, 2000) and as DNA-damaging agents that cause DNA rearrangements and point mutations (Prieto *et al.*, 2004). Nevertheless, enteric bacteria are intrinsically resistant to high concentrations of bile and individual bile salts (Gunn, 2000). *Salmonella* cultures can be adapted to resist extremely high bile

concentrations by previous exposure to a sublethal dose (van Velkinburgh & Gunn, 1999). The mechanisms involved in enterobacterial bile resistance are not fully understood. The isolation of mutants sensitive to bile has identified cellular functions required for bile resistance. The list includes a variety of envelope structures (Picken & Beacham, 1977; Prouty *et al.*, 2002; Ramos-Morales *et al.*, 2003), porins (Thanassi *et al.*, 1997), efflux pumps required for bile transport outside the cell (Ma *et al.*, 1994; Nikaido *et al.*, 1998; Thanassi *et al.*, 1997), regulatory functions (Sulavik *et al.*, 1997), the two-component system PhoPQ (van Velkinburgh & Gunn, 1999), DNA adenine methyltransferase (Dam) (Heithoff *et al.*, 2001; Pucciarelli *et al.*, 2002), and DNA repair functions (Prieto *et al.*, 2006).

Bile has also a role as an environmental signal. *Salmonella* can sense bile in the intestine and use it to down-regulate secretion and invasion genes of pathogenicity island 1 (Prouty & Gunn, 2000). Moreover, it has been shown that bile induces the formation of *Salmonella* biofilms on the surface of gallstones (Prouty *et al.*, 2003). The gallbladder is the niche for chronic *Salmonella* infections, which are in turn associated with gallstone formation and development of hepatobiliary carcinomas (Dutta *et al.*, 2000).

Intestinal colonization by *Salmonella* takes place in the ileum. The first step is penetration of the intestinal epithelial monolayer. *Salmonella* preferentially enters microfold (M) cells, which are specialized epithelial cells that sample intestinal antigens and transport them to lymphoid cells in the underlying Peyer's Patches [specialized lymphoid tissue in the small intestine (Jones *et al.*, 1994; Kohbata *et al.*, 1986)]. The mechanisms of infection of M cells remain poorly defined (Martinez-Argudo & Jepson, 2008). *Salmonella* type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 enables the manipulation of the cytoskeleton of intestinal epithelial cells through the injection of an array of bacterial effector molecules into the host cytoplasm (Galan & Zhou, 2000; Lostroh & Lee, 2001). *Salmonella* induces its uptake by initiating membrane ruffling which facilitates bacterial internalization by a process thought to resemble macropinocytosis (Cossart & Sansonetti, 2004). The invading *Salmonella* cells that have been translocated through the intestinal epithelial layer reach the subepithelial compartment where they interact with dendritic cells. In addition, *Salmonella* cells can also be internalized by resident macrophages (Hopkins *et al.*, 2000).

In gastroenteritis, the intestinal epithelium promotes an intense inflammatory response consisting largely of migration of polymorphonuclear leukocytes across the epithelial monolayer into the intestinal lumen (Day *et al.*, 1978; Kumar *et al.*, 1982; McGovern & Slavutin, 1979).

In enteric fever, systemic infection results from the ability of *Salmonella* to survive and replicate in mononuclear phagocytes (Hensel *et al.*, 1998). The ability to survive inside host cells is dependent on the SPI-2-encoded T3SS that injects effector proteins into host cells. Some such proteins have evolved to subvert the bactericidal properties of macrophages and to create a specialized *Salmonella*-containing vacuole in which they can replicate (Haraga *et al.*, 2008). Inside the macrophage, *Salmonella* cells disseminate through the blood stream accumulating in mesenteric lymph nodes, and ultimately in the spleen (Salcedo *et al.*, 2001).

Several pathogenicity islands have been described in *S.* Typhimurium, but SPI-1 and SPI-2 are the largest and most studied (Hensel, 2004). Other factors that contribute to virulence include pathogenicity islets (Groisman & Ochman, 1997), prophages (Figueroa-Bossi *et al.*, 2001), and plasmid-encoded virulence determinants, such as the *spv* genes of the virulence plasmid (Gulig *et al.*, 1993; Rotger & Casadesus, 1999).

Control of invasion involves a number of genetic regulators and environmental stimuli. The lumen of the distal small intestine is microaerobic with an osmolarity higher than 300 mOsm (Fordtran, 1968). The pH varies with diet, but the small intestine and the colon typically have pHs ranging from 6 to 7 (Argenzio & Southworth, 1975; Bohnhoff *et al.*, 1964; Cummings *et al.*, 1987). SPI-1 genes have been shown to be maximally expressed under low oxygen conditions (Bajaj *et al.*, 1996; Jones & Falkow, 1994; Russell *et al.*, 2004), high osmolarity (Bajaj *et al.*, 1996; Galan & Curtiss, 1990) and near-neutral pH (Bajaj *et al.*, 1996). In fact, *in vitro* conditions normally used to induce SPI-1 genes are pH7, rich medium with 0.3M NaCl, and microaerobiosis.

A number of compounds repress invasion at inappropriate times (e.g. in portions of the intestinal tract not productive for invasion, or within macrophages, after invasion has occurred). As described above, bile is secreted into the proximal small intestine and represses SPI-1 expression. Short-chain fatty acids (SCFAs) are produced by the anaerobes of the large intestine, particularly by members of the

genera *Lactobacillus* and *Bacteroides*. SCFAs concentration varies along the gastrointestinal tract. The highest levels are found in the colon, ranging from 130 to 300 mM total SCFAs, depending on the animal species and on the diet (Argenzio & Southworth, 1975; Bohnhoff *et al.*, 1964; Cummings *et al.*, 1987; Macfarlane *et al.*, 1992). High levels of two SCFAs, propionate and butyrate, inhibit growth of some pathogenic bacteria including *Salmonella* (Bohnhoff *et al.*, 1964; Meynell, 1963) and decrease invasion gene expression (Lawhon *et al.*, 2002). Similarly, cationic peptides and limiting Mg^{2+}, two conditions found within macrophages, are also known to reduce SPI-1 gene expression (Bader *et al.*, 2003; Garcia Vescovi *et al.*, 1996).

3. The virulence plasmid of *Salmonella enterica*

Virulence factors responsible for pathogenicity in enteric bacteria are often encoded by plasmids, as in *E. coli*, *Yersinia* spp. and *Shigella* spp. In *Salmonella* the existence of plasmid-borne virulence genes was first suggested in 1982; however, the contribution of virulence plasmids to pathogenesis in *Salmonella* is less important than in the aforementioned bacteria.

Salmonella virulence plasmids are 50 to 100 kb in size and have been called "serovar-specific plasmids" (Baumler *et al.*, 1998; Guiney *et al.*, 1994; Gulig *et al.*, 1993). Only a few serovars of *Salmonella enterica* belonging to subspecies I carry a virulence plasmid, particularly those showing host adaptation (*S.* Paratyphi C, Enteritidis, Dublin, Choleraesuis, Gallinarum / Pullorum, Abortusovis and Typhimurium). Moreover, not every isolate of a plasmid-bearing serovar carries a virulence plasmid (Boyd & Hartl, 1998).

All *Salmonella* virulence plasmids share a 7.8-kb region, *spv* (*Salmonella* plasmid virulence), required for bacterial proliferation in the mononuclear phagocytic system of warm-blooded vertebrates (Gulig *et al.*, 1993; Rotger & Casadesus, 1999). *spv* genes are also present on the chromosome of subspecies II, IIIa and IV. The limited sequence variation found in two genes of this locus suggests that the *spv* region is evolutionarily new and has been acquired by lateral transfer much more recently than other virulence genes of *Salmonella* (Boyd & Hartl, 1998). Other loci of the plasmid involved in the biosynthesis of fimbriae (*pef* or *fae*) and in serum resistance (*rsk*, *rcK* and *traT*) may play a role in other stages of the infection process (Guiney *et al.*, 1994; Gulig *et al.*, 1993).

Plasmid-cured strains are able to colonize and persist in the spleen and the liver, but bacterial growth is controlled by host defenses and infection does not develop. The virulence plasmid affects intracellular growth in macrophages, but not in non-phagocytic cells (Gulig *et al.*, 1998). Intraperitoneal LD_{50}s of wild-type and cured derivatives are not as different as they are peroral route. Curing of the viulence plasmid in *S.* Typhimurium SL1344 rises the oral LD_{50} in BALB/c mice 10^4 fold (Gulig & Curtiss, 1987).

The serovar-specific plasmid of *S.* Typhimurium contains a complete transfer operon that enables plasmid self-transmission. In other serovars, plasmids have often suffered deletions. Such plasmids are mobilizable if the origin of transfer, *oriT*, is present (Barrow & Lovell, 1989; Chu *et al.*, 2002; Ou *et al.*, 1994). The presence of some *tra* genes in the virulence plasmids of all serovars suggests that a *Salmonella* ancestor acquired the virulence plasmid by conjugation and that divergence has occurred during the evolution of the various serovars (Rotger & Casadesus, 1999).

Based on the genetic organization of the virulence plasmids shown in Figure I.1, it can be assumed that the ancestral virulence plasmid carried the IncFIIA replicon with the *spvRABCD* locus because these regions can be found in all the virulence plasmids (PSLT001–PSLT006 and PSLT027–PSLT051 loci using the *S.* Typhimurium nomenclature). The ancestor plasmid probably later fused with another replicon, IncFIB, containing *pef*, *srgAB* and *rck* (PSLT007–PSLT013, PSLT016–PSLT027). The IncFIB replicon inserted in between the IncFIIA origin of replication and the *ccdAB* genes, thus separating these regions. This insertion happened only in the ancestor of the current plasmid of *S.* Typhimurium, *S.* Enteritidis and *S.* Choleraesuis, as indicated by the fact that *S.* Gallinarum and *S.* Dublin virulence plasmids do not harbour the IncFIB origin of replication. Virulence plasmids of serovars Enteritidis and Choleraesuis could well be variants of the virulence plasmid of *S.* Typhimurium generated by deletion events (Beninger *et al.*, 1988; Buisan *et al.*, 1994; Lax *et al.*, 1990; Montenegro *et al.*, 1991; Rodriguez-Peña *et al.*, 1997; Rychlik *et al.*, 2006; Watson *et al.*, 1995).

The two independent replicons, *repB* and *repC*, are functional and control the low copy number of these plasmids (1–2 copies per chromosome) (Rodriguez-Peña *et al.*, 1997; Tinge & Curtiss, 1990a). Despite their low copy number, virulence plasmids are very stable owing to the *par* locus, which expresses the partition

function (Tinge & Curtiss, 1990b). In addition, the *Salmonella* virulence plasmid contains an addiction module homologous to the F sex factor *ccdAB* region, which in F is involved in plasmid stability (Engelberg-Kulka & Glaser, 1999). The mechanism of this toxin-antitoxin system has been described in the F plasmid. Upon plasmid loss, the antitoxin, CcdA, is degraded by the Lon ATP-dependent protease (Van Melderen *et al.*, 1994), whereas the toxin, CcdB, binds to the enzyme DNA gyrase and inhibits DNA replication (Bernard & Couturier, 1992; Miki *et al.*, 1992).

FIGURE I.1 Linear genetic maps of virulence plasmids of *S. enterica* subspecies I serotypes. Regions of homology shared among plasmids are shown as closed bars. Areas of the plasmid of *S. enterica* serotype Dublin that are not present in *S. enterica* serotype Typhimurium are shown as open bars. [Adapted from Baumler et *al* (Baumler *et al.*, 1998)].

An example of evolutive engineering in *Salmonella* clinical isolates is the emergence of hybrid virulence-resistance (VR) plasmids, resulting from an association between antimicrobial resistance determinants and specific virulence plasmids. VR plasmids all possess *spv*; however, they differ in the presence of other virulence determinants and in the antibiotic resistance gene profile (Guerra *et al.*, 2002; Mendoza Mdel *et al.*, 2009).

4. The virulence plasmid of *Salmonella enterica* serovar Typhimurium

The serovar-specific plasmid of S. *typhimurium* was designated pSLT by Jones *et al.* in 1982 (Jones *et al.*, 1982), who used S. *typhimurium* LT2 as reference strain. Nevertheless, this plasmid of 93 Kb had already been described by Dowman & Meynell in 1970 (Dowman & Meynell, 1970). Since its discovery, the virulence plasmid of *S.* Typhimurium was thought to be mobilizable but nonconjugative, despite having a complete transfer operon. In 1999, B. Ahmer and F. Heffron

showed that the virulence plasmids of ATCC 14028 and LT2 were indeed self-transmissible with mating conditions different than those previously used. However, these authors were not able to detect conjugation in the SL1344 strain (Ahmer *et al.*, 1999).

Plasmid classification has employed a variety of methods, none of which has become standard (Datta, 1977; Kline & Palchaudhuri, 1980; Seelke *et al.*, 1982). A classification based on plasmid incompatibility was developed in the early 1970s (Chabbert *et al.*, 1972; Richards & Datta, 1979; Sagai *et al.*, 1976; Sasakawa *et al.*, 1980), based on the possibility of stable simultaneous maintenance of two plasmids in a given host. Plasmids with similar replication control systems are defined as incompatible and cannot be maintained in the same host cell (Datta, 1979; Novick, 1987). There are about 30 plasmid incompatibility (Inc) groups (Couturier *et al.*, 1988). Another classification, based on the replication mechanism, classifies plasmids into rolling-circle-replicating (RCR) plasmids, theta-replicating plasmids, and plasmids that use a strand-displacement mechanism of replication (del Solar *et al.*, 1998; Espinosa *et al.*, 1995). Recently, the sequence of plasmid relaxases has been used to classify mobilizable and self-transmissible plasmids in six MOB families (Garcillan-Barcia *et al.*, 2009).

The virulence plasmid of *S.* Typhimurium is a F-like plasmid containing a complete and functional *tra* operon similar to that of the F plasmid. pSLT belongs to the MOB_{F1} relaxase classification. Other well-known F-like plasmids, such as R1, R100 (NR1) and F are also members of this family. The pSLT plasmid uses theta replication during vegetative growth and rolling-circle replication during conjugation (Lawley, 2004). The *repB* and *repC* replicons hybridize with IncFII and IncFI replicons, respectively. When the virulence plasmid of *S.* Typhimurium was tested for incompatibility, it was found to be compatible with plasmids of 23 different incompatibility groups, even with plasmids belonging to the IncFI and IncFII replicons (Ou *et al.*, 1990; Ou, 1993). Classification of *Salmonella* virulence plasmids into incompatibility groups is therefore unclear. The *S.* Typhimurium virulence plasmid is compatible with the virulence plasmids of *S.* Gallinarum/Pullorum but incompatible with virulence plasmids of serovars Enteritidis, Choleraesuis and Dublin (Ou *et al.*, 1990).

5. The transfer operon of IncF plasmids

Conjugative systems have been classified as a subset of type IV secretion systems (T4SS) (Lawley *et al.*, 2003b). T4SS encoded on Gram-negative conjugative elements are cell envelope-spanning complexes that are believed to form a pore or channel through which DNA and/or proteins travel from the cytoplasm of the donor cell to the cytoplasm of the recipient cell.

Conjugative pilus morphology and pilus-specific phage sensitivity classifications have suggested that pilus-dependent transfer systems might belong to two evolutionary families (Frost, 1993; Ippen-Ihler, 1991; Paranchych & Frost, 1988). One family includes the IncF plasmids and other plasmids with some F-like characteristics (e.g., IncD, IncC, and IncJ). The second family includes plasmids of IncP, IncN, IncW, and IncI incompatibility groups as well as the Ti plasmid of *Agrobacterium tumefaciens* (Kado, 1993; Lessl *et al.*, 1992).

The F plasmid of *Escherichia coli* is the paradigm of plasmid-specified transfer systems and has been the subject of study for many years (Willetts, 1993). All the functions required for the conjugative transmission are encoded on a 34 kb transfer region called *tra*.

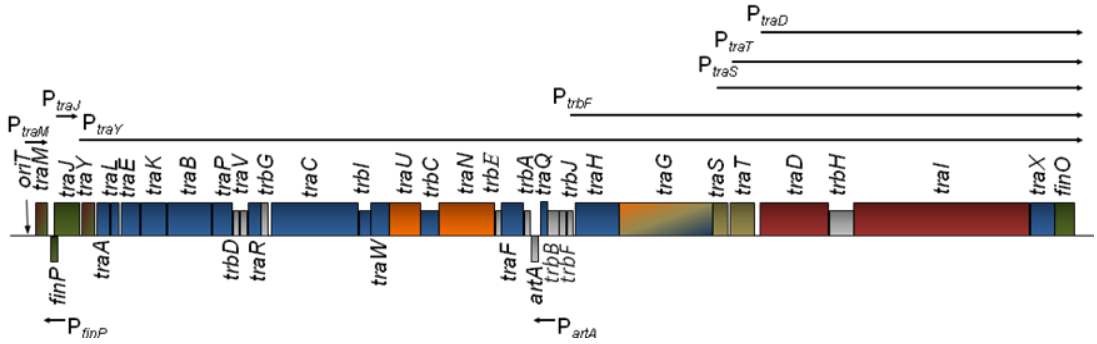

FIGURE I.2 Genetic map of the F-plasmid transfer region. Arrows denote transcripts initiating at the indicated promoters. The functional class associated with a particular gene is indicated by the color of its box: blue, pilus biogenesis; brown, surface or entry exclusion; orange, mating-aggregate stabilization; green, regulation; red, nicking and initiation of the DNA transfer; grey, unknown/nonessential function.

The distribution of loci within the *tra* region and the transcripts originated inside the region are indicated in Figure I.2. P_Y is the main promoter of the transfer operon. Additional promoters (P_F, P_S, P_T, and P_D) precede the *trbF*, *traS*, *traT* and *traD* genes, respectively. Maximal expression of the operon occurs when P_Y is active and stimulated by the TraJ activator protein. The transfer systems of most F-like

plasmids also express the regulatory genes *finO* and *finP*, whose products reduce expression of *traJ*. An IS3 insertion in the F *finO* gene is responsible for the constitutive transfer system characteristic of the F plasmid (Cheah & Skurray, 1986).

The *tra* operon includes genes involved in synthesis and assembly of pilus filaments, mating pair stabilization, surface exclusion, and transfer of DNA to the recipient. The functions of other loci such as *trbD*, *trbG*, *traR*, *trbE*, *trbA*, *trbB*, *trbJ*, *trbF*, *trbH* and *artA* remain unclear. TraP, TrbB, TrbI, and ORF169 are non-essential components of F-like T4SS (Anthony *et al.*, 1999; Frost *et al.*, 1994; Lawley *et al.*, 2003a). On the other hand, TraA, TraB, TraC, TraE, TraG (the N-terminal domain), TraK, TraL, and TraV proteins are widely conserved members of type IV secretion systems (see Table I.1), whereas TraF, TraG (C-terminal domain), TraH, TraN, TraU, TraW, TrbB, TrbC, and TrbI are involved in F-specific T4SS (Christie *et al.*, 2005; Lawley *et al.*, 2003b). TraQ is present only in T4SS F and close relatives like pSLT (Lu *et al.*, 2002).

Protein	P-type homolog	Size range (aa)	Cellular location(s)	Proposed function in F plasmid
TraA	TrbC/VirB2	112-128	IM, E	Pilin
TraB	TrbI/VirB10	429-475	IM-P	Pilus extension
TraC	TrbE/VirB4	799-893	IM*	Pilus tip formation
TraE	TrbJ/VirB5	130-261	IM	Pilus tip formation
TraG	TrbL/VirB6	912-1329	IM-P	Pilus tip formation; mating pair stabilization; entry exclusion
TraK	TrbG/VirB9	299-410	P/OM	Pilus tip formation
TraL	TrbD/VirB3	93-105	IM	Pilus tip formation
TraV	TrbH/VirB7	171-316	OM	Pilus extension

TABLE I.1 Summary of widely conserved members of type IV secretion systems. Hyphen (–) indicates that one part of the protein is in the first location and another part in the second location; slash (/) indicates that the protein can be find in the first and in the second location; asterisk (*) indicates that the protein is a peripheral inner membrane protein. Abbreviations: IM, inner membrane; E, extracellular; P, periplasm; OM, outer membrane; aa, amino acid.

The pilus is an extracellular filament produced by the donor cell, and creates a specific contact with one or more recipient cells. Once extended, F pili can depolymerize. Thus, recipient cells or phages to which these filaments have attached are carried to the donor cell surface as the pilus depolymerizes in cell-to-cell contacts, depolymerization leads to formation of a mating pair. Once the mating aggregate is stabilized, a single strand of DNA is transferred in a 5'-to-3' direction, beginning at the nicking site of the origin of transfer (*oriT*). The process of plasmid transfer results in recircularization of the transferred strand and synthesis of complementary DNA in the donor and recipient cells.

5.1. Synthesis and assembly of the pilus

The pilus filament is made of a single subunit (pilin) arranged in a helical array to give a fiber 8 nm in diameter with a 2-nm hollow core. The helix contains five subunits per turn. The length of the pilus can be variable, ranging from 1-2 μm to 20 μm depending on the growth conditions. Synthesis of the mature pilin subunit depends on three *tra* products expressed by the genes *traA*, *traQ* and *traX*.

traA encodes a 13-KDa propilin subunit. All F-like propilin subunits contain a long leader sequence that is either known or predicted to be cleaved by the host leader peptidase LepB to produce a 7-kDa peptide called pilin (Majdalani & Ippen-Ihler, 1996; Majdalani *et al.*, 1996). After removal of the signal sequence, the pilin subunit is oriented in the inner membrane with its N- and C-termini positioned in the periplasm (Harris *et al.*, 1999; Manchak *et al.*, 2002). The correct insertion and accumulation of pilin in the inner membrane require the TraQ chaperone-like inner membrane protein (Lu *et al.*, 2002). The pilin peptide is further modified by acetylation of its N-terminus by the inner membrane protein TraX to yield the mature F-pilin subunit (Maneewannakul *et al.*, 1995; Moore *et al.*, 1993).

Assembly of conjugative F-like pili on the bacterial surface requires some elements of the T4SS. Pilin subunits are stored as a pool in the inner membrane prior to assembly on the cell surface (Moore *et al.*, 1981) and pili are assembled by addition of pilin subunits to the base of the pilus (Maher *et al.*, 1993).

traK, *traE*, *traL*, *traC*, and the 5´ end of *traG* are involved in pilus tip formation on the cell surface, whereas *traB*, *traV*, *traW*, *traF*, and *traH* are involved in pilus extension (Anthony *et al.*, 1999; Frost *et al.*, 1994). The location and

structure of products expressed by the pilus assembly genes are presumably arranged to interact with one another to remove F pilin subunits from the inner membrane, polymerize them, and extend the F pilus from the cell surface (Anthony *et al.*, 1999; Frost *et al.*, 1994).

TraK shares similarity to secretin proteins (Deng & Huang, 1999). Although TraK is a periplasmic protein, it associates with the outer membrane in the presence of the F T4SS (Harris *et al.*, 2001). The C-terminal region of TraK contains two conserved domains. The β-domain is present in all secretins and is proposed to be embedded within the outer membrane to form a ring structure. The S-domain binds to a lipoprotein which serves as a periplasmic chaperone (Guilvout *et al.*, 1999). The C-terminus of TraK has been shown to interact with TraV, a lipoprotein that anchors TraK to the outer membrane, whereas the N-terminus of TraK interacts with TraB, an inner membrane protein (Harris *et al.*, 2001). The TraB-TraK-TraV complex likely forms an envelope-spanning structure similar to that of the Ti plasmid T4SS (Baron *et al.*, 2002).

The *traE* and *traL* genes are predicted to encode membrane proteins in which hydrophobic regions capable of spanning the membrane are linked by sequences that form turns and carry positively charged residues capable of interacting with the membrane surface (Frost *et al.*, 1984).

TraC is predicted to be a peripheral inner membrane protein with both Walker A and Walker B motifs, which energize pilus assembly (Cao & Saier, 2001; Rabel *et al.*, 2003).

TraG (938 aa in F) is one of the largest proteins encoded by the *tra* region (Frost *et al.*, 1994). This protein has three roles in conjugation: the N-terminal region is involved in pilus tip formation (Anthony *et al.*, 1999; Frost *et al.*, 1994); aa 610-673 region is involved in entry exclusion of DNA into the recipient cell (Audette *et al.*, 2007); and the entire protein participates in mating pair stabilization (Firth & Skurray, 1992).

The TraB protein is predicted to contain an N-terminal anchor in the inner membrane with the bulk of the protein located within the periplasm. The N-terminal region contains coiled-coil domains, which are probably involved in multimerization, and a proline-rich domain, suggesting an extended structure (Gilmour *et al.*, 2003).

The proline-rich domain, by analogy with other such motifs, could interact with SH3 domains in other proteins, an interaction central to signal transduction (Bliska, 1996). In R27, it has been shown that the homolog of TraB, TrhB, interacts with itself and with the coupling protein (TraD in F), linking the relaxosome to the T4SS (Gilmour *et al.*, 2003; Harris *et al.*, 2001). The TraB-TraK-TraV interaction may create a continuous pore from the cytoplasm through the cell envelope to the extracellular environment (Figure I.3).

TraV-like proteins are lipoproteins located in the outer membrane, and contain two conserved cysteines thought to be involved in multimerization. TraV has been shown to interact with TraK (Harris *et al.*, 2001) and to anchor TraK to the outer membrane (Arutyunov *et al.*, 2010; Harris *et al.*, 2001). TraV is probably also the anchor protein for TraF, TraH, TraU, and TraW, which appear to be periplasmic proteins that associate with the outer membrane when the transfer apparatus is complete (Arutyunov *et al.*, 2010) (Figure I.3).

TraF might have chaperone-like activity and shares similarity to the thioredoxin superfamily. TraF of F and pSLT have a thioredoxin-like domain but lacks the C-X-X-C motif (Elton *et al.*, 2005). However, TraF appears to have a function other than disulfide bond chemistry since TraF is essential for conjugation.

TrbB is included in the thioredoxin superfamily containing the thioredoxin like domain with the C-X-X-C motif, this protein appears to be a periplasmic disulfide bond isomerase (Elton *et al.*, 2005). TrbB likely plays a role in thiol redox chemistry and might help F T4SS proteins such as TraH, TraU, and TraN, which have 6, 10, and 22 conserved cysteines, respectively, achieve the correct conformation for assembly into the transferosome complex (Elton *et al.*, 2005); however, TrbB is not essential for conjugation since there seems to be redundancy in function between TrbB and the *E. coli* chromosomaly-encoded thioredoxin-like DsbC.

TrbC is fused to the N-terminus of TraW in R27, Rts1, R391 and SXT, whereas TraW and TrbC are separate proteins in F, pED208 and pNL1. The fusion of TrbCF to TraWF suggests that the functions of these proteins are linked.

TraH contains C-terminal coiled-coil domains, suggesting the formation of higher order structures, either with other TraH molecules or with other components

of the T4SS. Interestingly, yeast two-hybrid analysis has shown that TraF, TraH, TraU, TraW, TrbB, and TrbI form an interaction group, with TraH directly linked to TraF, TraU, and TrbI (Harris & Silverman, 2004). TraH is thought to interact indirectly with TraW via TraU (Harris & Silverman, 2004).

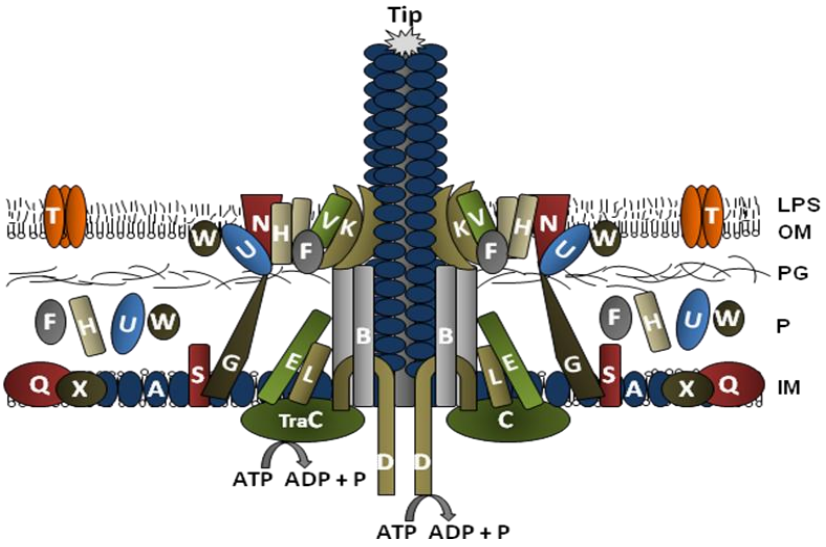

FIGURE I.3 A representation of the F-like T4SS transfer apparatus drawn from available information. The pilus is shown assembled with five TraA (pilin) subunits per turn extending from the inner membrane through a putative secretin-like outer membrane pore involving TraK. TraK is anchored by TraV and interacts with TraB. TraB interacts with the coupling protein, TraD, creating a continuous pore from the cytoplasm through the cell envelope to the extracellular environment. [Adapted from Lawley et *al* (Lawley *et al.*, 2003b)]. Abbreviations: LPS, lipopolysaccharide; OM, outer membrane; PG, peptidoglycan; P, periplasm; IM, inner membrane.

5.2. Mating pair stabilization

The interaction between the pilus tip of the donor and a receptor on the recipient cell surface results in specific contacts between mating pairs or mating aggregates (since more than two cells might be involved) (Achtman, 1975). After the initial contact, the pilin subunits depolymerize into the membrane, drawing the cells together to form a conjugation bridge for the passage of DNA (Durrenberger *et al.*, 1991).

Mating pairs are difficult to break apart prematurely and require significant force to do so. They are resistant to disruption by mechanical forces or the addition of SDS, which allows cells to mate more efficiently in liquid media (Achtman, 1977; Bradley *et al.*, 1980; Manning *et al.*, 1981).

The products of *traN* and *traG* may stabilize mating contacts at an early stage of the interaction between donor and recipient cell surfaces. Both of them are responsible for the phenotypes of resistance to SDS and shear forces (Achtman *et al.*, 1977). TraU is proposed to be involved in DNA transfer. Mutations in *traU*, *traG* and *traN* have similar phenotype (Moore *et al.*, 1990).

The whole of TraG, but especially the C-terminal region, is involved in mating pair stabilization. The C-terminal region has been proposed to interact with TraN to stabilize mating pairs (Firth & Skurray, 1992). However, this interaction remains unproven (Firth & Skurray, 1992; Klimke *et al.*, 2005). Alternatively, it has been proposed that TraG might interact with TraU (Audette *et al.*, 2007).

TraN appears to act as an adhesin. It is a cysteine rich outer-membrane protein that interacts with OmpA. The OmpA outer membrane protein in the recipient has been implicated in mating-pair formation with F donors in liquid media, but not on solid surfaces (Achtman, 1978; Klimke & Frost, 1998; Klimke *et al.*, 2005; Manoil & Rosenbusch, 1982; Manoil, 1983; Morona *et al.*, 1984). Other F-like TraN proteins do not necessarily require OmpA (Skurray *et al.*, 1974). Thus, the role of OmpA in conjugation remains in question and mating-pair formation may occur in different ways on solid and in liquid media. The N- and C-terminal regions of TraN proteins are highly conserved whereas the central region displays extensive divergence. It is this central region that is involved in OmpA recognition by TraN as well as TraN multimerization (Klimke & Frost, 1998). TraN is predicted to have an ATP-binding domain (Maneewannakul *et al.*, 1992), but it is unknown whether mating-pair stabilization is ATP dependent.

5.3. Surface exclusion

There are two processes involved in the prevention of redundant DNA transfer between donor cells. The first is surface exclusion, which blocks the initial steps in mating-pair formation (Achtman *et al.*, 1977; Harrison *et al.*, 1992; Klimke *et al.*, 2005). The second is entry exclusion, which blocks DNA transfer after a mating pair has been established (Kingsman & Willetts, 1978).

The *traT* gene encodes a 26 kDa precursor protein which is modified by the addition of a diglyceride and two fatty acids. The cleavage after residue 21 gives a

polypeptide of 23.8 kDa, and further acylation completes the mature protein (Minkley, 1984; Perumal & Minkley, 1984). It has been shown that different plasmids that carry small changes in the *traT* sequence define the specificity of TraT in surface exclusion (Harrison *et al.*, 1992). The TraT protein has two hydrophobic segments which could span the outer membrane. It forms a multimeric structure in the membrane (Minkley & Willetts, 1984), which is visualized by electron microscopy as a five-membered structure. The precise role of TraT in surface exclusion is unclear. One possibility is that the amino acids that define TraT specificity may extend into the core of the ring and be recognized by the pilus tip of the homologous plasmid (Harrison *et al.*, 1992). This protein also confers serum resistance, contributing to bacterial virulence (Sukupolvi & O'Connor, 1990).

Entry exclusion is a plasmid-specific process, defined by TraS in the inner membrane of the recipient cell (Jalajakumari *et al.*, 1987). DNA transport is blocked between cells carrying the same conjugative plasmids (Kingsman & Willetts, 1978; Ou, 1975) by TraG in the donor recognizing its cognate TraS in the recipient (Anthony *et al.*, 1999; Marrero & Waldor, 2005). The mechanism of action of TraS remains an enigma. TraS blocks the signal for DNA transfer between donor cells (Achtman *et al.*, 1977; Achtman *et al.*, 1980; Kingsman & Willetts, 1978). It requires an intact peptidoglycan in the donor cell, suggesting a role for the cell wall in signal transduction. TraG from the donor could be translocated to the recipient cell where it might interact with TraS instead of its true receptor.

5.4. Nicking and initiation of transfer of DNA to the recipient

The initiation of DNA transfer is thought to involve recognition of a mating signal by the DNA transfer machinery, which is known as the relaxosome. The proteins involved in DNA metabolism during transfer are TraM, TraY, TraI, and TraD (Everett & Willetts, 1980).

It has been suggested that TraM may process the signal indicating that a mating pair has been formed (Willetts, 1987), and that TraY may direct the nicking enzyme, TraI, to the origin of transfer (*oriT*) (Traxler & Minkley, 1988). TraI, which is DNA helicase I, apparently has two functions, nicking and unwinding (Reygers *et al.*, 1991), whereas TraD is involved in pumping the single DNA strand into the

recipient cell in a 5´-to-3´ direction (Ohki & Tomizawa, 1968; Panicker & Minkley, 1985).

The *oriT* region is located upstream *traM*, and contains the site where nicking occurs (Ihler & Rupp, 1969). The first locus transferred to the recipient cell is ORF169, which is part of the leader region, and *tra* genes are transferred last. The *oriT* can be divided into two sections: one is essential for nicking, and the second is essential for transfer (Fu *et al.*, 1991). The nick site in the complementary strand of an AC-rich region is highly conserved among plasmids of the F-like family. Downstream, there are two integration host factor (IHF) recognition sites, and several binding sites for TraY and TraM. Differences in these proteins and differences in their recognition sites determine the specificity of the *oriT*s. Figure I.4 shows a diagram of the transfer regions in R100 and F.

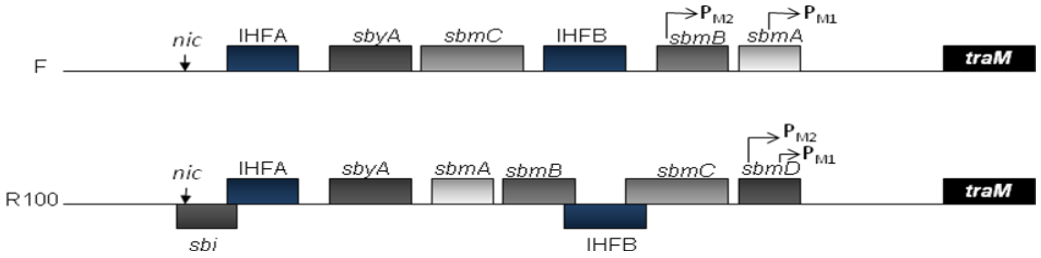

FIGURE I.4 Diagram of the binding sites in *oriT* of the F and R100 plasmids. P_{M1} and P_{M2} refer to promoters for the two *traM* transcripts. *sbm* are binding sites for TraM; *sby* are binding sites for TraY; IHF are the binding sites for IHF; and *nic* is the site where the nick is done.

TraM is essential for mating but its specific function has not been clarified. It has been proposed to be involved in mating signal transduction. This protein is able to bind specific DNA sequences. There are several binding sites for TraM between *oriT* and *traM*, some of them inside its own promoter. Binding of TraM to its promoter represses the expression of *traM* (Abo & Ohtsubo, 1993; Lu *et al.*, 2003; Penfold *et al.*, 1996; Schwab *et al.*, 1993). Another TraM-binding site maps between the binding sites of TraY and IHF (Figure I.4) (Abo & Ohtsubo, 1995; Di Laurenzio *et al.*, 1995; Fekete & Frost, 2002). When TraM is bound to this region, a higher efficiency of nicking is observed (Fekete & Frost, 2000; Kupelwieser *et al.*, 1998). TraM is a cytoplasmic protein with a small segment that seems to be associated to the inner membrane. The N-terminal region of TraM is responsible for DNA binding in a plasmid-specific manner (Kupelwieser *et al.*, 1998). Because this protein is able

to bind to *oriT* and to the membrane, TraM has been proposed to anchor the plasmid to the transfer apparatus. It has been shown that, in R100, TraM interacts with TraD (Disque-Kochem & Dreiseikelmann, 1997). On the other hand, TraM is also involved in regulation (see below).

TraY is a specific-DNA binding protein that binds to the *sbyA* region, close to the nick site. The *oriT* region undergoes bending as a consequence of both its sequence and the action of TraY, TraM and IHF proteins bound to their cognate sites, which confer a particular conformation in this locus enabling TraI binding (Fekete & Frost, 2000; Howard *et al.*, 1995; Karl *et al.*, 2001). Besides its function in the relaxosome complex, TraY binds its own promoter (the main promoter of the operon) and plays a role in *tra* regulation.

TraI, also known as helicase I, has an essential role in *oriT*-related events. The N-terminal domain has a chain dependent transesterase activity. This protein catalizes a cutting reaction in one strand of DNA, so that 5´ DNA becomes covalently joined to TraI. The nick is done inside a sequence called *ssi* (single-strand initiation) or *nic,* and requires binding of IHF and TraY proteins to *oriT*. Despite this sequence is highly conserved among different plasmids, it carries differences that confer specificity. Nicking the specific single-strand is the first step required in transfer. The C-terminal domain of TraI has an ATP-dependent-helicase activity that unwinds DNA in a 5'-to-3' direction (Wilkins, 1993). TraI could also be involved in the correct termination of transfer, due to its ability to bind the 3´ end of one strand containing the *ssi* region to the 5´ strand covalently bound to TraI (Fukuda & Ohtsubo, 1997; Sherman & Matson, 1994).

TraD is an inner membrane protein, and may be involved in the active transport of DNA from the donor to the recipient. There are three reasons for this assumption: i) TraD is able to bind DNA nonspecifically; ii) TraD has ATP binding motifs; and iii) TraD is able to interact with TraM when it is bound to the *oriT* region (Disque-Kochem & Dreiseikelmann, 1997; Llosa *et al.*, 2002).

5.5. Leading region of transfer

The leading-region sequence, upstream the *oriT*, is the first to enter the recipient cell during conjugation. This portion is highly conserved among F-like

plasmids but it is not essential for transfer. This region encodes at least eight polypeptides (Cram *et al.*, 1984; Jackson *et al.*, 1984; Ray & Skurray, 1983). The functions of several genes of the leading region have been identified.

ORF169 (formerly gene *X*) is the first gene that is transferred to the recipient during conjugation. It is transcribed in the opposite orientation to *traM* and its function remains unknown. Although lack of this gene decreases the mating frequency 10 fold, the mutants are not affected in pilus synthesis, mating-pair formation or nicking of *oriT*, so it has been suggested that this putative membrane protein could facilitate the passage of the DNA transferred chain.

The *ssb* gene encodes a single-stranded-DNA-binding protein able to complement mutations in the *E. coli* chromosomal *ssb* product (Chase *et al.*, 1983; Kolodkin *et al.*, 1983). The chromosomal SSB product has a relevant role in the replication of DNA. The function of the plasmidical SSB is not clear, but it may play a role in establishing the newly transferred plasmid DNA in the recipient cell.

The gene *psiB* encodes a protein which inhibits the coprotease activity of RecA (and, therefore, the SOS response). *psiB* of F has been shown to be expressed in the recipient cell after transfer (Bagdasarian *et al.*, 1992; Jones *et al.*, 1992). However, *psiA*, which appears to be co-expressed with *psiB*, may not be involved in suppressing the SOS response in the recipient cell (Bagdasarian *et al.*, 1986; Bailone *et al.*, 1988; Loh *et al.*, 1989).

The *flm* sequence is largely identical to the *hok-sok-mok* region of plasmid R1 (Gerdes *et al.*, 1990). This plasmid maintenance system may also play a postconjugational role in ensuring the predominance of plasmid-carrying cells in the recipient population.

Certain sequences in the leader region are involved in synthesis of the complementary DNA strand once the transferred strand has entered the recipient cell. *ssiE* and *ssiD* (single-strand initiation) are the sites for this function. *ssiD* contains a promoter that is functional in single-stranded DNA. RNA polymerase synthesizes the RNA primer, which is necessary for synthesis of DNA by DNA polymerase III (Manwaring *et al.*, 1999; Masai & Arai, 1997). The requirement for RNA primer synthesis to initiate replacement strand synthesis in the donor *in vivo* argues against simple rolling-circle replication from the free 3'-OH, and suggests a

more complex mechanism for initiating replacement strand synthesis (Kingsman & Willetts, 1978; Willetts & Wilkins, 1984).

6. Regulation of the *tra* operon in incF family plasmids

In F-like plasmids, synthesis of pili and conjugal transfer are regulated by a variety of plasmid- and host- encoded factors.

6.1. Plasmid-encoded regulators

The plasmid-encoded regulators are TraJ, the FinOP system, TraM, and TraY (Frost *et al.*, 1994).

TraJ is a transcriptional activator of the main promoter of *tra* operon, P_Y (Finnegan & Willetts, 1972; Frost *et al.*, 1994). TraJ is plasmid specific, suggesting that TraJ variants encoded by different *traJ* alleles could recognize different sequences. The TraJ DNA-binding domain resembles the consensus sequence for DNA-binding proteins with helix-bend-helix motifs (Takeda *et al.*, 1983). In 1998, it was shown that TraJ of R100 was able to bind a specific sequence of DNA (*sbj*) between the end of *traJ* and the beginning of *traY* (Taki *et al.*, 1998). This region has binary symmetry and is physically close to the *traY* promoter (nucleotide -78 with respect to the transcription start). The sequences of *traJ* and *traY* are not highly conserved among the plasmids but contain sequences with the same type of symmetry (Frost *et al.*, 1994).

In most F-like plasmids, *traJ* is subject to postranscriptional regulation via the FinOP antisense RNA system (van Biesen & Frost, 1994). This phenomenon was first described in R100 because its frequency of conjugation was lower than the frequency of F, and F transfer was inhibited by the presence of the R100 plasmid in the donor cell. This phenotype of R100 was called Fin+ (fertility inhibition) (Womble & Rownd, 1988). Later on it was described that two factors were involved in this phenotype, FinP and FinO, and that this system was common among F-like plasmids. Several F-like plasmids, such as F and some R plasmids, are $FinO^-$, thus permitting TraJ synthesis (Cheah & Skurray, 1986). Other plasmids like R100 and pSLT have an active fertility inhibition system. FinP is plasmid-specific while FinO is interchangeable (Finnegan & Willetts, 1972; Finnegan & Willetts, 1971).

The *finP* gene encodes an antisense RNA, FinP, that negatively regulates TraJ expression. Antisense RNA-mediated gene regulation has been implicated in the control of a variety of prokaryotic cellular processes ranging from the control of copy number of accessory elements to the regulation of septum formation during cell division (Tetart & Bouche, 1992).

FinP is complementary to part of the 5'- untranslated region of the *traJ* mRNA (Dempsey, 1987; Finlay *et al.*, 1986; Mullineaux & Willetts, 1985). The secondary structure of the *traJ* leader includes three stem-loops named SLI, SLII and SLIII (Figure I.5). In turn, FinP RNA contains two stem-loops, SLI and SLII, complementary to those of *traJ* mRNA. The ribosome-binding site (RBS) and the two first codons of *traJ* are located in SLI (van Biesen *et al.*, 1993). Therefore, the binding of FinP to the 5´-UTR of *traJ* mRNA is believed to sequester the RBS within a FinP/*traJ* mRNA duplex via base pairing with the anti-RBS. When the duplex FinP-*traJ* mRNA is formed, it is rapidly degraded by the endoribonuclease III (RNAse III) (Jerome *et al.*, 1999) and TraJ translation is prevented, thus repressing expression of the transfer operon (Koraimann *et al.*, 1991; Koraimann *et al.*, 1996; Mullineaux & Willetts, 1985). RNAse III activity makes it difficult to detect complete *traJ* mRNA in a FinOP active system (Dempsey, 1987; Finnegan & Willetts, 1973; van Biesen *et al.*, 1993; Willetts, 1977).

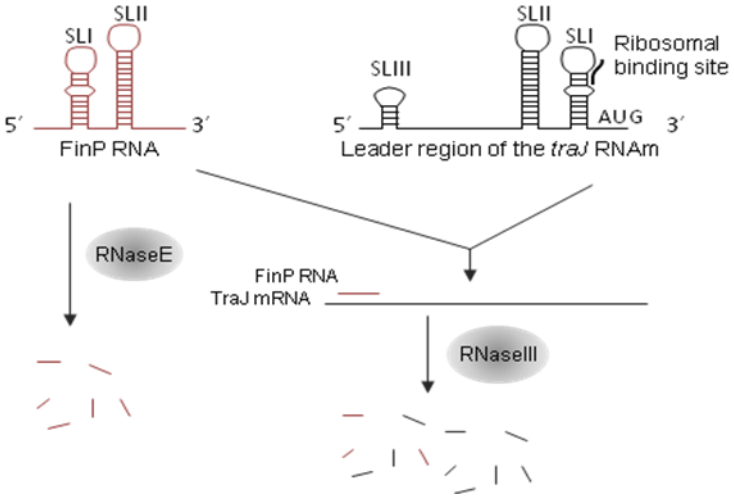

FIGURE I.5 Secondary structure diagram of FinP RNA and the leader region of *traJ* mRNA. Endorribonuclease E (RNAse E) cleaves the FinP single-stranded spacer between SL-I and SL-II, whereas endoribonuclease III (RNAse III) degrades the duplex FinP-*traJ* mRNA.

Contact between loops of RNA stem-loop structures is commonly the first interaction to occur during the process of RNA/RNA duplex formation. F-like conjugative plasmids encode eight different alleles of FinP with high variability in the loops (Finlay *et al.*, 1986; Frost *et al.*, 1994; Jerome *et al.*, 1999). The loop sequences of FinP and *traJ* mRNA are therefore thought to be responsible for mediating the plasmid specificity of the F-like FinOP systems (Finlay *et al.*, 1986; Koraimann *et al.*, 1991; Koraimann *et al.*, 1996) and are thought to be the initial site of interaction between the sense and antisense RNAs. Although the loop sequences of FinP in F-like plasmids vary, a common motif, 5´-YUNR-3´ (where Y represents pyrimidine, N is any base, and R is purine), is found in several *finP* alleles (Frost *et al.*, 1994), which is a key structural motif in the loops of many antisense RNA molecules (Franch *et al.*, 1999).

In contrast to other known antisense RNA systems, FinP-mediated repression occurs only in the presence of a protein, FinO (Finnegan & Willetts, 1973), which is encoded at the distal end of the transfer region (Finnegan & Willetts, 1973; Frost *et al.*, 1989). FinO is a basic cytoplasmic protein with a highly α-helical structure (Ghetu *et al.*, 2000). FinO has two functions: to increase the half life of FinP and to facilitate formation of the duplex FinP RNA:*traJ* mRNA.

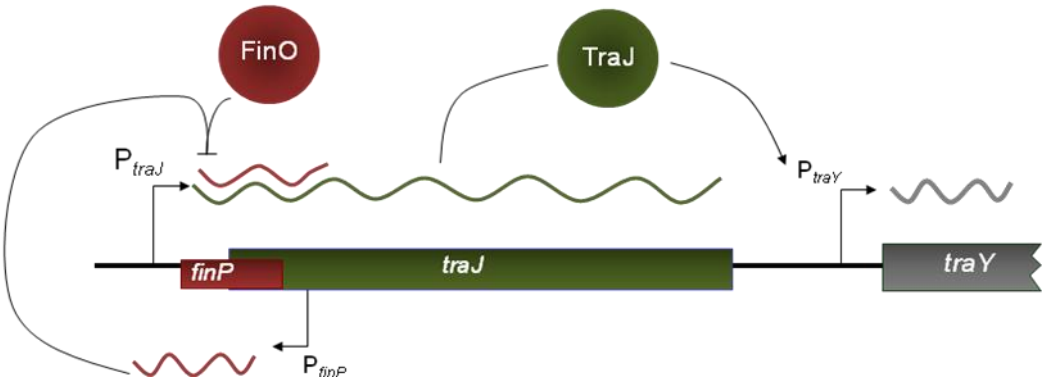

FIGURE I.6 Diagram of the fertility-inhibition system. Wave-shaped lines represent RNA. Promoter positions and transcription direction are indicated by straight arrows. Arrows denote activation and blunt lines denote inhibition.

FinO binds the relatively unstable FinP molecule (Ghetu *et al.*, 1999; Ghetu *et al.*, 2000; Jerome & Frost, 1999) and sterically inhibits RNAse E cleavage of the single-stranded spacer between SL-I and SL-II (Jerome *et al.*, 1999; Sandercock & Frost, 1998), allowing the steady state concentration of FinP to increase to sufficient

levels (approximately ten fold more in $FinO^+$ than $FinO^-$ strains) to mediate repression of *traJ* expression (Frost *et al.*, 1989; Lee *et al.*, 1992). Indeed, the requirement of FinO for inhibition of transfer and *traJ* expression can be alleviated by providing FinP at elevated copy number in the cell (Koraimann *et al.*, 1991; Koraimann *et al.*, 1996). The concentration of FinO in cells containing R100 appears to be extremely low (van Biesen & Frost, 1992) and cells with *finO* present at high copy number are rather unstable [data not shown from the paper (van Biesen & Frost, 1994)].

FinO catalyzes FinP/*traJ* mRNA duplex formation *in vitro,* which is believed to allow rapid sequestration of the *traJ* RBS and efficient inhibition of *traJ* expression *in vivo* (Koraimann *et al.*, 1991; Koraimann *et al.*, 1996) (Figure I.6). The duplex RNA/RNA formation rate increases five fold in presence of FinO (van Biesen & Frost, 1994). In the absence of FinO, FinP represses the expression of a *traJ*::*lacZ* translational fusion about six fold (Koraimann *et al.*, 1996). In the presence of FinO, repression increases up to 400 fold. Hence, the process is dose-dependent and repression of *traJ* mRNA translation depends on the concentrations of FinP and FinO. FinO is able to bind FinP RNA or *traJ* mRNA separately (Gubbins *et al.*, 2003; Jerome & Frost, 1999; Sandercock & Frost, 1998; van Biesen & Frost, 1994), and it has been proposed that this ability could facilitate the first contact between the two molecules (Gubbins *et al.*, 2003). The N-terminal region of FinO has been shown to destabilize double-strand RNA at the same time of catalizing duplex RNA-RNA formation (Gubbins *et al.*, 2003). Initial binding of FinO to FinP and *traJ* mRNA would bring them closer, destabilizing the RNA loop structures SLI and SLII, and facilitating duplex formation between the two molecules.

The TraY protein, encoded by the first gene of the *tra* operon, is a sequence-specific DNA-binding protein with binding sites in cognate *oriT* and P_Y promoter regions (Inamoto & Ohtsubo, 1990; Nelson *et al.*, 1993). In F plasmid, TraY activates transcription of its own promoter (Figure I.7) (Penfold *et al.*, 1996; Silverman & Sholl, 1996), whereas in R100, the binding of TraY represses its own transcription (Stockwell *et al.*, 2000; Taki *et al.*, 1998). TraY has also been identified as a positive regulator of *traM* transcription in F as well as in R1 and R100 (Penfold *et al.*, 1996; Stockwell *et al.*, 2000). Owing to the TraY binding site in *oriT* is close to *traM* promoter, it is thought that TraY could activate directly the transcription of *traM*.

As indicated above, TraM is required to transmit the signal for the initiation of DNA transfer. In addition, TraM has a regulatory role as autorepressor (Figure I.7). Another function was suggested because in R100 *traM* transcripts end inside the coding region of *traJ* (Dempsey, 1989). It has been proposed that these transcripts offer an additional amount of *traJ* leader, helping to decrease the levels of free FinP and subsequently permit translation of *traJ*. Furthermore, an increase in the *traM* transcription level correlates with higher transcription of *tra* operon, (Stockwell *et al.*, 2000). It has been shown that in R100-1 (FinO$^-$) maximal frequencies of plasmid transfer require expression of *traM* in *cis* with respect to t*raJ* (Dempsey, 1994). This hypothesis does not apply to F because the end of the *traM* transcripts is not inside *traJ*.

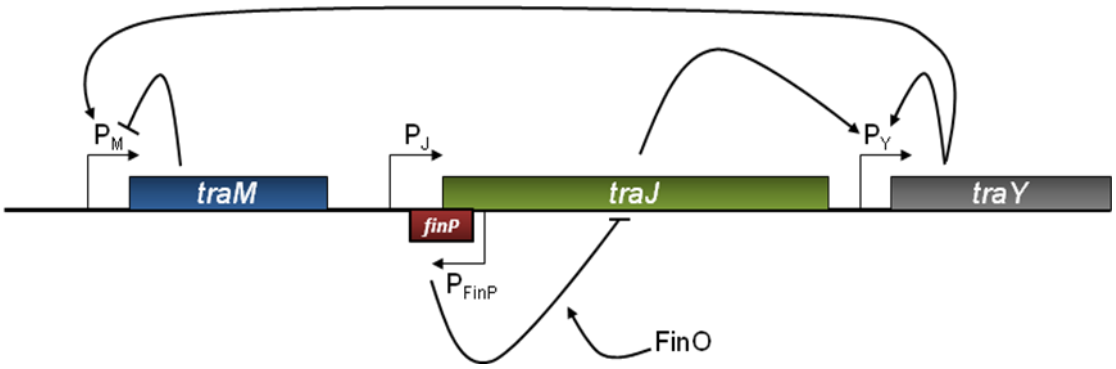

FIGURE I.7 Schematic representation of the plasmidic regulatory circuit of F *tra* region. Positive effects are indicated by lines ending in arrowheads, whereas black bars indicate negative regulatory effects. P_M, P_J, P_{finP} and P_Y indicate the *traM*, *traJ*, *finP* and *traY* promoters respectively. [Adapted from Gubbins et *al* (Gubbins *et al.*, 2002)].

6.2. Host-encoded regulators

In F-like plasmids, conjugation and synthesis of pili are also regulated by host-encoded factors, such as the general regulators ArcA, IHF, H-NS, CRP, Lrp, GroEL, and Hfq, the metabolism-related factors Cpx, FlhDC, FruR, GcvA or Sdh, and a DNA modification function (Dam methylation). Some regulators appear to play analogous functions in different plasmids, while others may be plasmid-specific. For instance, CRP (cAMP receptor protein) is an activator of conjugal transfer in pRK100 (Starcic *et al.*, 2003) but not in pSLT (A. Serna, E.M. Camacho, and J. Casadesús, unpublished results). Another example involves the nucleoid

protein H-NS, which acts as a repressor of conjugal transfer in F upon entry into stationary phase (Will *et al.*, 2004) but activates mating functions in plasmid pRK100 (Starcic-Erjavec *et al.*, 2003). Hence, F-like plasmids share a common regulatory design, but specific-plasmid responses to host-encoded regulators are also found. The latter may reflect different host-adaptation strategies and/or individual responses to physiological signals. This view is supported by the observation that optimal conditions for plasmid transfer vary from plasmid to plasmid. For instance, in aerobiosis, transfer of the *Salmonella* virulence plasmid is repressed in rich medium (Ahmer *et al.*, 1999), a trait not found in the F sex factor. In turn, the sudden decrease in F plasmid transfer upon entry into stationary phase (Frost & Manchak, 1998) is not observed in pSLT (Camacho *et al.*, 2005b).

Adaptation of bacterial cells to environmental changes is facilitated through a large number of two-component signal transduction systems. In such systems environmental signals are sensed by a membrane-associated kinase and phosphatase, and the information is then transduced to a cognate response regulator which is typically activated by phosphorylation. The activated response regulator stimulates or represses transcription of its target genes and thereby generates a cellular response (Hoch, 1995). ArcAB and CpxAR are two-component signal transduction systems involved in conjugation.

The ArcA protein is a response regulator of a two-component system that senses the redox state of the bacterial cell and allows adaptation to changes in environmental O_2 concentrations. The sensor of this system is ArcB. Approximately 120 operons are regulated directly or indirectly by ArcAB (Liu & De Wulf, 2004). In response to microaerobiosis, the sensor kinase phosphorylates ArcA, which then represses transcription of genes involved in respiratory metabolism and stimulates fermentative-metabolism-related genes (Lynch & Lin, 1996). ArcA seems to have two functions in the control of bacterial conjugation: Arc (aerobic respiratory control) and Sfr (sex factor regulation). ArcA activates *tra* transcription, especially under microaerobiosis. ArcA binds upstream the main *tra* promoter (P_Y) (Serna *et al.*, 2010; Silverman *et al.*, 1991b; Strohmaier *et al.*, 1998; Taki *et al.*, 1998) and the binding site is conserved among F-like plasmids (Strohmaier *et al.*, 1998). Binding of ArcA to DNA changes its topology. In fact, *in vitro tra* transcription requires a supercoiled DNA (Gaudin & Silverman, 1993), suggesting that ArcA could introduce the negative supercoiling necessary for activating the promoter. In aerobiosis, ArcA activates the *traY* promoter of F-like plasmids in an ArcB-independent manner

(Silverman *et al.*, 1991a), but the ArcB sensor is necessary for activation of P_Y in microaerobiosis (Serna *et al.*, 2010).

The CpxAR two-component system senses and responds to extracytoplasmic stresses, including overexpression of the outer membrane lipoprotein NlpE (Snyder *et al.*, 1995), overexpression of misfolded P-pilus subunits (Jones *et al.*, 1997), and elevated pH (Nakayama & Watanabe, 1995), among others. Misfolded proteins in the cell envelope are thought to be the main activating signal of the Cpx system in *E. coli* (Raivio *et al.*, 2000). The stress-inducing signal is transferred from CpxA to its cognate cytoplasmic response regulator, CpxR (Hoch, 1995; Weber & Silverman, 1988). The phosphorylated active CpxR upregulates transcription of several genes involved in protein folding and protein degradation in the bacterial envelope (Danese *et al.*, 1995; Dartigalongue & Raina, 1998; Jones *et al.*, 1997). Gain-of-function mutants, *cpxA**, are characterized by their ability to suppress the toxic effects of mislocalized and misfolded proteins in the cell envelope (Cosma *et al.*, 1995). *cpxA** mutants exhibit numerous and varied phenotypes, including resistance to amikacin (Rainwater & Silverman, 1990), sensitivity to high temperature (McEwen & Silverman, 1980), and tolerance to alkaline pH (Danese & Silhavy, 1998). F transfer is sensitive to cell envelope stress. The *cpxA** mutation reduces F plasmid conjugation by postranscriptional inactivation of the positive activator, TraJ, via the HsIVU protease in a phase-dependent manner, since the induction of Cpx in mid-log phase cells does not appreciably alter TraJ levels (Gubbins *et al.*, 2002).

The integration host factor, IHF, is a nucleoid-associated protein found in *E. coli*, *Salmonella enterica* and related bacteria (Azam & Ishihama, 1999; Dorman & Deighan, 2003). IHF contributes to genome organization (Oppenheim *et al.*, 1993; Pettijohn, 1996) and participates in the control of DNA transactions such as transcription (Goosen & van de Putte, 1995), replication (Ryan *et al.*, 2002), site-specific recombination (Bushman *et al.*, 1985; Dorman & Higgins, 1987; Eisenstein *et al.*, 1987; Esposito *et al.*, 2001) and transposition (Crellin *et al.*, 2004; Makris *et al.*, 1990). IHF binds to a conserved sequence and bends DNA by angles of up to 180° (Rice *et al.*, 1996; Rice, 1997). This DNA-bending activity is critical to the role played by ArcA in a variety of systems due to its ability to promote long-range interactions (Goosen & van de Putte, 1995). The intracellular concentration of IHF is growth-phase dependent and increases at the onset of the stationary phase (Ali Azam *et al.*, 1999; Aviv *et al.*, 1994; Bushman *et al.*, 1985; Ditto *et al.*, 1994; Murtin

et al., 1998; Weglenska *et al.*, 1996). IHF of *S. enterica* and *E. coli* is an heterodimeric protein whose subunits are encoded by the *ihfA* and *ihfB* genes. However, homodimers also have DNA-binding activity (Hiszczynska-Sawicka & Kur, 1997; Werner *et al.*, 1994; Zablewska & Kur, 1995; Zulianello *et al.*, 1994).

In *S.* Typhimurium, IHF is required for the expression of all three type III secretion systems: SPI-1, SPI-2 and the flagellar secretion system (Mangan *et al.*, 2006). It is also involved in the regulation of genes for adaptation to the stationary phase of growth (Mangan *et al.*, 2006), indicating a role in coordinating regulation of the pathogenic traits with adaptation to the stationary phase. IHF binds two sites in both the F (Tsai *et al.*, 1990) and R100 (Dempsey & Fee, 1990; Inamoto, 1990) *oriT* regions. Intrinsic bends and bends induced by IHF (Tsai *et al.*, 1990) are proposed to fulfill the three-dimensional structural requirements at *oriT* necessary for cleavage at *nic* by the plasmid-encoded relaxase, TraI. In the F plasmid, IHF and TraY are required for the nicking reaction *in vitro* (Nelson *et al.*, 1995) and similar characteristics have been shown for the closely related plasmid R100 (Abo & Ohtsubo, 1995; Fukuda & Ohtsubo, 1997).

H-NS is one of the most intensively studied members of the family of bacterial nucleoid-associated proteins. H-NS has a preference for binding to DNA that is A+T-rich and intrinsically curved (Grainger *et al.*, 2006; Lucchini *et al.*, 2006; Navarre *et al.*, 2006; Oshima *et al.*, 2006). It has been suggested that this specificity may serve as a defensive mechanism against foreign genetic material without losing the benefit of future usage of this material if necessary (Dorman, 2007). H-NS is detectable at an approximately constant level throughout growth (Free & Dorman, 1995; Hinton *et al.*, 1992) and can also bind RNA (Williams *et al.*, 1996). Genetic evidence has indicated that H-NS is a domainin: in other words, a protein that helps to close the looped DNA domains in the nucleoid (Hardy & Cozzarelli, 2005; Noom *et al.*, 2007). H-NS has an oligomerization domain in the N-terminal region, a nucleic acid-binding domain in the C-terminal region, with an unstructured and flexible linker connecting them (Cusick & Belfort, 1998; Dorman, 2004; Rimsky, 2004). The oligomerization domain is critical for dimer formation (Lang *et al.*, 2007). These activities lead themselves to transcription repression, the principal regulatory activity of H-NS. The large-scale repression that is associated with H-NS is analogous to gene silencing in eukaryotes, as originally proposed by Bernt Uhlin 20 years ago (Göransson *et al.*, 1990). H-NS represses the transcription

of hundreds of genes in Gram-negative bacteria, including *S.* Typhimurium (Stoebel *et al.*, 2008).

Although instances of transcriptional activation directly mediated by H-NS are relatively uncommon (Hommais *et al.*, 2001), H-NS has been shown to act as an activator of *traJ* expression in the F-like plasmid pRK100 (Starcic-Erjavec *et al.*, 2003). In the F sex factor, H-NS appears to be pivotal in down-regulating F transfer as the host cell enters stationary phase. H-NS is a transcriptional repressor of *traJ* and *traM*, and also binds to intrinsically curved DNA at the P_Y promoter, overlaping the binding site of ArcA. This binding alters local supercoiling and represses *tra* transcription (Will & Frost, 2006b). Interestingly, TraJ is not required for *tra* operon expression in an *hns* mutant (Will & Frost, 2006b) suggesting that TraJ serves at least in part to counter the repressive effects of H-NS at the promoter (Will & Frost, 2006b). In the case of pSLT, H-NS has two differential functions: repression of transfer in a non-phase-dependent manner, which agrees with the pSLT capacity to promote conjugation in stationary phase (Camacho *et al.*, 2005a); and repression of *finP* transcription in a Dam^- background (see below) (Camacho & Casadesus, 2005; Camacho *et al.*, 2005b).

Hfq (host factor required for phage Qβ RNA replication) regulates many aspects of RNA biology, influencing stability, translation, and RNA bacteriophage replication, often via small RNAs (Gottesman, 2004; Valentin-Hansen *et al.*, 2004). This protein was first identified as a bacterial protein necessary for the replication of the RNA plus-strand of bacteriophage Qβ (Franze de Fernandez *et al.*, 1968; Franze de Fernandez *et al.*, 1972; Miranda *et al.*, 1997). An *hfq* null mutant of *E. coli* shows pleiotropic effects, including reduced growth rates, increased sensitivity to ultraviolet light, mutagens and oxidants, and increased cell length. Moreover, an *hfq* mutation alters the levels of more than 50 proteins, due in part to the requirement of Hfq for efficient translation of the *rpoS* gene (Brown & Elliott, 1996; Muffler *et al.*, 1997). Hfq also binds proteins, including RNase E and PNP, which are involved in mRNA mediated decay (Folichon *et al.*, 2003; Mohanty *et al.*, 2004; Morita *et al.*, 2005; Morita *et al.*, 2006). Hfq forms hexamers that preferentially bind sequences of AU-rich RNA, often flanked by structured regions (Moller *et al.*, 2002; Zhang *et al.*, 2002). Hfq is a relatively abundant protein (Ali Azam *et al.*, 1999) whose expression profile during the growth cycle is far from well known (Ali Azam *et al.*, 1999; Tsui *et al.*, 1997; Vytvytska *et al.*, 2000). Hfq has no role in FinOP-mediated repression (Will & Frost, 2006a); otherwise, the IS3 insertion in the F *finO*

gene (Cheah & Skurray, 1986) would not increase so much F plasmid transfer. Instead, Hfq appears to act as a repressor of TraJ and TraM synthesis by destabilizing the corresponding transcripts (Will & Frost, 2006a). Hfq binds to the intergenic UTR, 3´ to *traM* and 5´ to *traJ*, and decreases the stability of transcripts containing this region (Will & Frost, 2006a). This is another case of Hfq/H-NS overlaping in the same regulatory circuit, such as it happens in the regulation of *rpoS*, *hns* or *bgl* (Dole *et al.*, 2004; Hengge-Aronis, 1996; Lease *et al.*, 1998; Sledjeski & Gottesman, 1995; Sledjeski *et al.*, 2001).

GroEL is the most exhaustively studied chaperone, a member of the Hsp-60 family of promiscuous type-I chaperons found in prokaryotes and in eukaryotic mitochondria. It has been estimated that up to 5% of the proteins in *E. coli* depend on GroEL to fold (Kerner *et al.*, 2005). A set of 85 substrates of GroEL have been identified as being unable to fold in the absence of GroEL/ES (Kerner *et al.*, 2005). For the majority of these proteins, aggregation emerges as the main obstacle to folding (Niwa *et al.*, 2009). In these cases, GroEL simply provides a safe environment in which proteins can fold without associating with other proteins (Agard, 1993; Ellis & Hemmingsen, 1989; Ellis, 2003; Hayer-Hartl *et al.*, 1996; Horwich *et al.*, 2009; Mayhew *et al.*, 1996; Weissman *et al.*, 1996). In other cases GroEL accelerates folding by periodically disrupting or destabilizing off-pathway misfolded states (Corrales & Fersht, 1996; Jackson *et al.*, 1993; Lin *et al.*, 2008; Shtilerman *et al.*, 1999; Sparrer *et al.*, 1997; Todd *et al.*, 1994; Todd *et al.*, 1996; Weissman *et al.*, 1994). In R1, GroEL is involved in down-regulation of T4SS gene expression and conjugation via TraJ (Zahrl *et al.*, 2007). An increase of temperature from 22°C to 43°C reduces R1 *tra* operon expression to undetectable levels and reduces conjugative transfer by 2 to 3 orders of magnitude. These effects reflect the interaction of GroEL with TraJ, which is followed by rapid degradation of the TraJ protein (Zahrl *et al.*, 2007).

Deoxyadenosyl methyltransferases are common in bacteria, and most of them are part of restriction/modification systems (Cheng, 1995). Dam is a methylase of enteric bacteria (Herman & Modrich, 1982). In *Escherichia coli* and related genera, Dam methylation provides signals to initiate chromosome replication (Messer *et al.*, 1985), to direct chromosome segregation (Ogden *et al.*, 1988), to target the daughter strand for mismatch repair (Glickman *et al.*, 1978), and to regulate the expression of certain genes. For instance, in *S.* Typhimurium Dam methylation activates the pathogenicity island SPI-1 (Balbontin *et al.*, 2006; Lopez-

Garrido & Casadesus, 2010), while it represses the *std* fimbrial operon (Balbontin *et al.*, 2006; Jakomin *et al.*, 2008). Owing to these multiple roles, Dam methylation is an important factor in cellular welfare, and its loss causes pleiotropic defects (McGraw & Marinus, 1980; Peterson & Mount, 1993; Prieto *et al.*, 2004; Pucciarelli *et al.*, 2002; Torreblanca & Casadesus, 1996) including extreme bile sensitivity (García-del Portillo, 1999; Heithoff *et al.*, 1999; Heithoff *et al.*, 2001; Pucciarelli *et al.*, 2002) and impairing of virulence (García-del Portillo, 1999; Heithoff *et al.*, 1999).

Dam binds to DNA nonspecifically as a monomer, moving by linear diffusion and specifically methylating adenosines of 5´-GATC-3´ sequences. Dam shows high processivity for most DNAs; that is, after one methylation event, it slides on the same DNA molecule and carries out additional methylation events. This high processivity effectively increases the rate of Dam methylation and may reflect the fact that there are few (<100) Dam molecules present in a single *E. coli* cell, yet there are about 19,000 GATC sites to methylate. Dam levels vary according to growth rate (Lobner-Olesen *et al.*, 1992). Dam-regulated genes can be classified into two classes: genes whose expression is coupled to the cell cycle; and genes that contain GATC sites in upstream regulatory sequences. In such cases, the methylation state of one or more GATC sites affects the affinity of a regulatory protein for its DNA binding site.

In the 1990s, a screen for genes regulated by Dam methylation in *S.* Typhimurium identified the transfer operon of pSLT as a Dam-repressed locus (Torreblanca & Casadesus, 1996). Derepression of *tra* in a Dam$^-$ background results in increased frequencies of conjugal transfer, a phenomenon also observed in other plasmids of the F-like family such as F and R100 (Camacho *et al.*, 2005a; Torreblanca *et al.*, 1999). In pSLT, Dam methylation does not act directly on the *tra* operon but acts on the regulatory genes *traJ* and *finP* (Camacho & Casadesus, 2002; Torreblanca *et al.*, 1999). Transcription of *traJ* is repressed by Dam methylation (Camacho & Casadesus, 2005) (see below). In turn, transcription of *finP* is activated by Dam methylation (Camacho & Casadesus, 2005; Torreblanca *et al.*, 1999). This dual effect of Dam methylation accounts for the increase in *tra* operon expression observed in Dam$^-$ donors (Camacho *et al.*, 2005b). Transcription of the pSLT *finP* gene occurs at reduced rates in *dam* mutants (Camacho & Casadesus, 2005; Torreblanca *et al.*, 1999). Repression of *finP* transcription in a Dam$^-$ background is exerted by the nucleoid protein H-NS (Camacho & Casadesus, 2005).

However, the different expression levels of the *finP* gene in Dam+ and Dam- strains cannot be explained by a local effect of Dam methylation upon H-NS binding because Dam-mediated repression is still observed in a mutant *finP* promoter lacking the GATC site that overlaps the -10 module (Camacho & Casadesus, 2005). The involvement of upstream DNA sequences is likewise discarded by deletion analysis (Camacho & Casadesus, 2005). Hence, H-NS-mediated repression of *finP* may reflect a condition or state that occurs in Dam$^-$ mutants but not in the wild type. Tentative explanations might be that a higher H-NS concentration exists in *Salmonella* Dam$^-$ mutants, as reported for *E. coli* (Oshima *et al.*, 2002), or that lack of methylation favors a change in the pattern of H-NS association to the cell nucleoid. Because N^6 methylation at individual GATC sites is known to influence local DNA structure (Diekmann, 1987), it seems conceivable that the methylation state of thousands of GATCs might influence nucleoid organization and potentially affect H-NS binding. Support for this hypothesis was obtained by microarray analysis of gene expression in *E. coli* overexpressing Dam (Lobner-Olesen *et al.*, 2003).

Lrp (leucine-responsive regulatory protein) is a global regulator in prokaryotes (Bell & Jackson, 2000; Brinkman *et al.*, 2002; Brinkman *et al.*, 2003). In *Salmonella* and *E. coli*, Lrp is a small DNA-binding protein, which binds to DNA in a cooperative manner (Brinkman *et al.*, 2003). L-leucine influences the oligomeric state of Lrp (Chen *et al.*, 2001; Chen & Calvo, 2002; de los Rios & Perona, 2007; Peterson *et al.*, 2007). In some cases leucine is required as an effector (Lin *et al.*, 1992), while in others leucine has no effect *in vivo* (Ernsting *et al.*, 1992). Lrp is negatively autoregulated (McFarland & Dorman, 2008) and influences the expression of genes whose products are involved in amino acid biosynthesis and degradation, nitrogen metabolism, carbohydrate degradation, synthesis of fimbriae, and transport (Newman & Lin, 1995). It can also affect site-specific recombination (Kelly *et al.*, 2006; Roesch & Blomfield, 1998). Moreover, Lrp has unique roles in *Salmonella*. These include repression of *spv* virulence genes (Marshall *et al.*, 1999) and other key virulence regulatory genes of SPI-1 and SPI-2 (Baek *et al.*, 2009).

High levels of *lrp* expression depend on the accumulation of ppGpp (guanosine-3´,5´-bisphosphate). In turn, the intracellular level of ppGpp increases with energy or amino acid starvation (Cashel, 1996). Lrp is able to bind to DNA unspecifically, and has been tentatively considered a DNA organizer protein (Chen *et al.*, 2001; D'Ari *et al.*, 1993). Binding of Lrp to DNA requires sequences with some

binary symmetry, high levels of AT, and specific nucleotides at certain places (Calvo & Matthews, 1994; Newman, 1996; Shultzaberger & Schneider, 1999). The promoter targets normally contain several binding sites for Lrp (Brinkman *et al.*, 2003), and cooperative binding to such sites induces DNA curvature (Calvo & Matthews, 1994). Lrp is required for *traJ* transcription, and hence for *tra* operon expression (Camacho & Casadesus, 2002). Two Lrp binding sites, LRP-1 and LRP-2 upstream the *traJ* promoter, are necessary for transcriptional activation (Camacho & Casadesus, 2005). LRP-2 contains a GATC site whose methylation state affects Lrp binding: methylation in both DNA strands decreases the affinity of Lrp for the LRP-2 site, while efficient Lrp binding occurs to a non-methylated GATC site. The effect of hemimethylation is strand-specific. This asymmetry supports a model in which passage of the replication fork may permit Lrp-mediated activation of conjugal transfer in one daughter plasmid molecule but not in the other (Camacho & Casadesus, 2005). Furthermore, because the pSLT strand transferred during conjugation is the noncoding strand, the active epigenetic state of *traJ* may be transmissible to the recipient cell: use of the incoming DNA strand as template will reproduce the methylation pattern that permits *traJ* activation, and the recipient cell will instantly become a donor if sufficient Lrp is available.

Recent genetic screens have described new metabolic factors involved in conjugal transfer of pSLT. Lack of succinate dehydrogenase causes a >20 fold increase in pSLT transfer in aerobiosis, but has little effect under microaerobiosis (Serna *et al.*, 2010). Succinate dehydrogenase, encoded by genes of *sdhCDAB* operon, is an enzyme which catalyzes succinate oxidation to fumarate and is involved in tricarboxylic acid (Krebs) cycle (Ackrell, 1992; Hederstedt & Rutberg, 1981). Transcription of the *sdhCDAB* operon is active in aerobiosis and repressed in anaerobiosis. ArcA binds the *sdhCDAB* promoter and represses transcription of the operon, especially in anaerobiosis. Sdh inhibits conjugal transfer in rich medium by reducing *traJ* transcription, probably in an indirect manner (Serna *et al.*, 2010). Hence, the ArcAB system of *S. enterica* stimulates pSLT transfer under microaerobiosis by activation of the *tra* operon and repression of the *sdhCDAB* operon (Serna *et al.*, 2010).

FruR or Cra (catabolite represor/activator), FlhDC (the major regulator of flagellum synthesis), and GcvA (a LysR-like transcriptional regulator) are repressors of pSLT transfer in rich medium, probably by controlling *traJ* transcription (Serna, A. and Casadesús, J., unpublished data).

7. Appendix

7.1. Biosynthesis of L-cysteine from inorganic sulfate

S. Typhimurium and *E. coli* can utilize a number of sulfur-containing compounds as sole sulfur source, including sulfate, sulfite, thiosulfate, sulfide, glutathionine, lanthionine, taurine and L-djenkolate [*S,S´*- methylene bis(cysteine)] as well as cysteine and cystine (formed by the oxidation of two cysteine residues covalently liked with a disulfide bond) (Kredich, 1996; Peck, 1961; van der Ploeg *et al.*, 1997). However, these organisms cannot utilize methionine as sole sulfur source (Kredich, 1996).

Synthesis of L-cysteine in *S.* Typhimurium is achieved by reaction of sulfide with O-acetylserine, or by reaction of thiosulfate with O-acetylserine, the latter to yield S-sulfocysteine which is then converted to cysteine (Figure I.8) (Kredich, 1987).

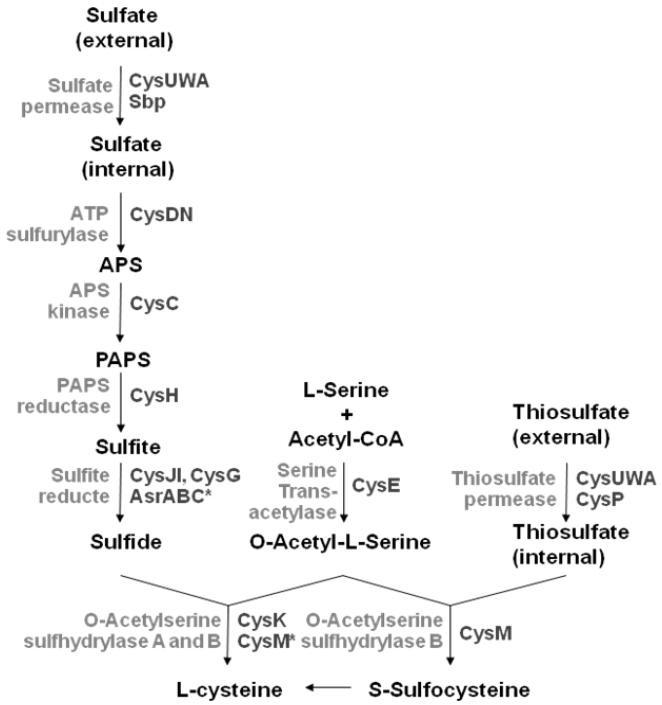

FIGURE I.8 Pathway of L-cysteine biosynthesis in *S.* Typhimurium and *E. coli*. Enzymatic activities are indicated on the left. Protein names according to the genes that encode them are indicated on the right. The asterisk (*) denotes that those proteins are utilized under anaerobic conditions. Abbreviations: APS, adenosine 5´-phosphosulfate; PAPS, 3´-phosphoadenosine 5´-phosphosulfate.

Biosynthesis of L-cysteine from inorganic sulfate and L-serine proceeds along a branched convergent pathway. On one arm sulfate is reduced to sulfide, while on the other L-serine is acetylated to O-acetylserine (Figure I.8). The group of genes whose function is involved in L-cystine transport and in the synthesis of L-cysteine from inorganic sulfate is called "the cysteine regulon" (Baptist & Kredich, 1977; Kredich, 1987). Operons and single genes involved in the cysteine biosynthetic pathway include *cysPUWAM*, *cysJIH*, *cysDNC*, *cysQ*, *cysK, cysE, cysG* and *spb*.

Most genes required for cysteine biosynthesis (*cysPUWAM*, *cysJIH*, *cysDNC*, *cysK* and *spb*) are positively regulated by *cysB*, which encodes a transcriptional activator belonging to the LysR family of regulatory proteins. The CysB protein binds immediately upstream the -35 region of cognate promoters. In the presence of inducer, CysB facilitates the formation of a transcription initiation complex (Monroe *et al.*, 1990; Ostrowski & Kredich, 1989; Ostrowski & Kredich, 1990). O-acetylserine spontaneously isomerizes to N-acetylserine (Sturgill *et al.*, 2004), which is a more potent inducer than O-acetylserine (Kredich, 1987; Kredich, 1992; Lynch *et al.*, 1994). CysB also represses its own synthesis by binding to the *cysB* promoter (Ostrowski & Kredich, 1991). Inducers stimulate CysB to bind sites involved in positive regulation and inhibit CysB binding to the negatively autoregulated *cysB* promoter. Sulfide and thiosulfate provide additional regulation as anti-inducers by antagonizing binding of O- and N- acetylserine to CysB (Hryniewicz & Kredich, 1991; Ostrowski & Kredich, 1990). Cysteine downregulates the pathway by inhibiting serine transacetylase, encoded by *cysE*, which synthesizes O-acetylserine (Kredich & Tomkins, 1966; Kredich *et al.*, 1969). Growth with poor sulfur sources results in maximal derepression of the sulfur regulon and rapid growth on rich media results in moderate induction (Anton, 2000). In general, expression of cysteine regulon requires CysB, the presence of inducer, and sulfur limitation (Kredich, 1992).

Most of the cysteine (Cys-SH) equivalents in the extracellular space exist as the oxidized form, cystine (Cys-S-S-Cys) due to the oxidizing environment of the extracellular space (Mansoor *et al.*, 1992). In contrast, because a reducing environment is maintained inside the cell, cysteine is predominantly intracellular (Arner & Holmgren, 2000). L-cystine transport into *S.* Typhimurium is mediated by three separate transport systems: CTS-1, CTS-2 and CTS-3 (Baptist & Kredich, 1977). CTS-1 is the main transport system and is repressed by growth on cysteine and other reduced sulfur sources. CTS-2 and CTS-3 are lower-capacity transporters

of cystine than CTS-1, although CTS-2 has higher affinity for cystine (Baptist & Kredich, 1977). Sulfate and thiosulfate are imported by the same permease as encoded by the genes *cysU-cysW-cysA* but having different periplasmic binding proteins, *sbp* and *cysP*, respectively (Hryniewicz & Kredich, 1991).

After entry into the cell, sulfate reduction requires activation by adenosine triphosphate (ATP) to yield adenosine 5´-phosphosulfate (APS) and pyrophosphate (PPi) (Robbins & Lipmann, 1958). This reaction is catalyzed by ATP sulfurylase, encoded by *cysD* and *cysN* (Robbins & Lipmann, 1958). The product of the *cysC* gene, APS Kinase, further phosphorylates APS to give 3´phosphoadenosine 5´phosphosulfate (PAPS) (Robbins, 1956), a toxic intermediate which is then enzymatically reduced by PAPS sulfotransferase (or PAPS reductase) to sulfite (Krone *et al.*, 1991; Tsang, 1983). Thioredoxin or glutaredoxin are the physiological reductants for the reaction (Tsang & Schiff, 1978; Tsang, 1981). During aerobic growth, reduction of sulfite to sulfide is catalyzed by NADPH-sulfite reductase, encoded by *cysG cysJ* and *cysI* (Siegel *et al.*, 1973). Under anaerobic conditions, *S.* Typhimurium expresses AsrABC, the anaerobic sulfite reductase (Hallenbeck *et al.*, 1989; Huang & Barrett, 1991). A mutant lacking this function accumulates sulfite from sulfate, implying that sulfite is a normal intermediate in assimilatory sulfate reduction (Dreyfuss, 1963). The final step consists of the formation of L-cysteine from O-acetylserine and sulfide. In *S.* Typhimurium this reaction is catalyzed by two isozymes: O-acetylserine sulphydrylase A and B, encoded by *cysK* and *cysM*, respectively. CysK is produced at high levels and is thought to be the primary enzyme responsible for this reaction (Kredich, 1996); however, CysM is the predominant enzyme catalyzing the reaction in anaerobic conditions (Kredich, 1996). The enzyme encoded by *cysQ* is thought to degrade a toxic intermediate of this pathway (Neuwald *et al.*, 1992).

7.2. The *cysDNC* operon

cysD is the first gene of the *cysDNC* operon. This operon encodes ATP sulfurylase and APS kinase. ATP sulfurylase is composed of two subunits: a catalytic subunit of 35 kDa encoded by *cysD* (Kredich, 1983) and a 53 kDa subunit homologous to GTP-binding proteins encoded by *cysN* (Leyh *et al.*, 1988; Leyh *et al.*, 1992). The native enzyme is a tetramer of CysD-CysN heterodimers (Liu *et al.*, 1994). *cysC* encodes the 22-kDa APS kinase (Leyh *et al.*, 1988; Leyh *et al.*, 1992;

Satishchandran *et al.*, 1992). This subunit is phosphorylated by ATP at Ser-109, and exists as a dimer in the phosphorylated state and mostly as a tetramer in the unphosphorylated state (Satishchandran & Markham, 1989).

The characteristics of *cys* promoters are similar to those of other positively regulated genes: their -10 regions resemble the TATAAT consensus sequence of σ^{70}-dependent promoters, while their -35 regions tend to differ significantly from the consensus TTGACA. Regulation of these promoters is often achieved by binding of an activator protein immediately upstream the -35 region, perhaps facilitating the formation of the transcription initiation complex (Hoopes, 1987; Raibaud & Schwartz, 1984). DNA binding, footprinting and *in vitro* transcription run-off experiments in the *S.* Typhimurium *cysJIH, cysK* and *cysP* promoters support this hypothesis (Hryniewicz & Kredich, 1991; Monroe *et al.*, 1990; Ostrowski & Kredich, 1989).

The -10 region of the *cysD* promoter (TATAGT) is closely related to the -10 consensus sequence, while the -35 region TTCATT diverges from the -35 consensus (Malo & Loughlin, 1990). A comparison between *E. coli* K-12 and *S.* Typhimurium LT2 and a subsequent analysis using prediction programs shows that *S.* Typhimurium might have two possible +1 start sites, one identical to the *E. coli* +1 and another further upstream (Figure I.9).

In *E. coli*, a possible CysB binding site has been proposed to exist in the *cysDNC* promoter (Malo & Loughlin, 1990) at approximately the same position of CysB recognition sites for positive transcriptional regulation in *cysJIH*, *cysK* and *cysP* promoters (Kredich, 1992). Although binding occurs with CysB alone, transcription initiation requires the additional presence of N-acetylserine, which stimulates binding to these promoters (Kredich, 1987; Kredich, 1992; Lynch *et al.*, 1994). *In vitro* experiments with *S.* Typhimurium have shown that sulfide and thiosulfate reverse the effects of N-acetylserine upon binding of CysB to *cys* promoters and upon transcription initiation as well (Hryniewicz & Kredich, 1991; Ostrowski & Kredich, 1990). These effects are specific for *cys* promoters and can be overcome by increasing the concentration of N-acetylserine. Thus, sulfide and thiosulfate are competitive anti-inducers. Sulfate, sulfite, cysteine and cystine do not have anti-inducer effects *in vitro*. The ability of the latter two compounds to prevent derepression by exogenous N-acetylserine *in vivo* has been believed to be mediated through the sulfide generated in large amounts in cystine-grown cells by

the inducible enzyme cysteine desulfhydrase, which degrades cysteine to pyruvate, ammonia, and sulfide (Kredich *et al.*, 1972). However, cysteine it is also known to downregulate the pathway by inhibiting the enzyme which synthesizes the inducer O-acetylserine (Kredich & Tomkins, 1966; Kredich *et al.*, 1969).

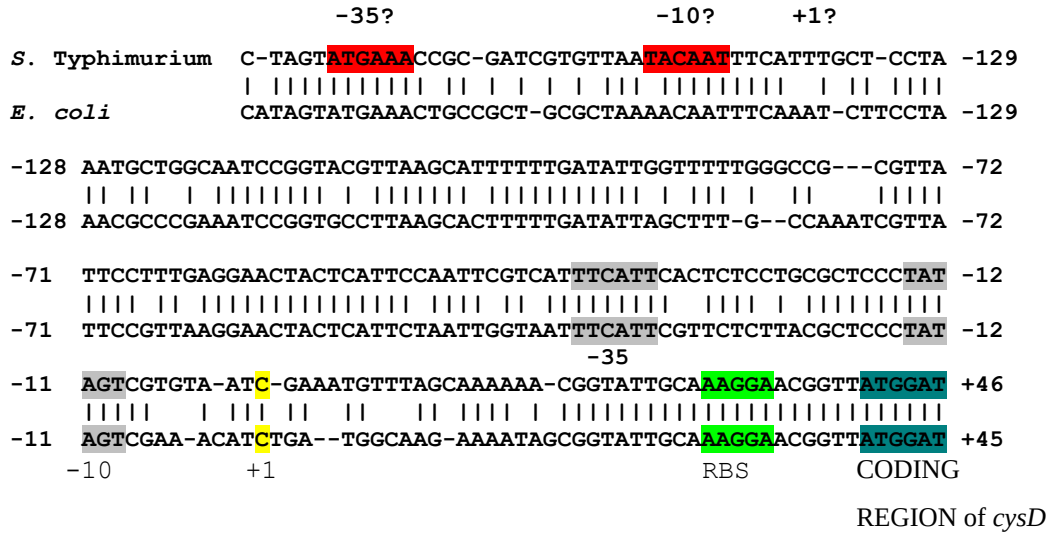

FIGURE I.9 Comparison between the *cysD* upstream region of *E. coli* K-12 and *S.* Typhimurium LT2. Red color indicates possible -10 and -35 regions in *S.* Typhimurium.

7.3. Physiological roles of L-cysteine

L-cysteine can serve as the sole source of carbon, nitrogen (Gutnick *et al.*, 1969) and sulfur (Peck, 1961) for *S.* Tyhpimurium. In other microorganisms, L-cysteine is known to perform other roles besides its function in metabolism. In *Bordetella pertussis* the availability of cysteine regulates toxin expression (Bogdan *et al.*, 2001), and genes of the sulfur assimilatory pathway are required for *Brucella melitensis* virulence (Lestrate *et al.*, 2000). In *Mycobacterium tuberculosis,* sulfur metabolism has been implicated in virulence, antibiotic resistance and anti-oxidant defense, and the *cysDNC* genes are induced upon exposure to oxidative stress, suggesting that sulfur assimilation is regulated in response to toxic oxidants (Pinto *et al.*, 2004). Biofilm formation by *E. coli* occurs at an accelerated rate in *cysB* (Ren *et al.*, 2005) and *cysE* mutants (Sturgill *et al.*, 2004). In a *cysE* mutant, addition of cysteine reduces biofilm formation 2.5 fold, suggesting that cysteine might be a signal that inhibits biofilm development (Sturgill *et al.*, 2004).

In *S.* Typhimurium, mutations in *cysB* and *cysE* confer resistance to mecillinam, and thiosulfate, sulfite, sulfide and cysteine abolish this resistance

(Oppezzo & Anton, 1995). *S.* Typhimurium can swarm on nutrient-rich media supplemented with a rich carbon source and containing an agar concentration of 0.5% to 0.8% (Burkart *et al.*, 1998; Kim & Surette, 2003; Toguchi *et al.*, 2000). *cysB* and *cysE* mutants fail to swarm unless cysteine is added to the medium, indicating that L-cysteine biosynthesis is crucial for complete swarm-cell differentiation (Turnbull & Surette, 2008).

L-cysteine has also a role in the intestine, one of the natural environments of *Salmonella* inside the host. The non-immune intestinal barrier relies on epithelial integrity, mucus production, and the presence and equilibrium of commensal bacteria. The mucous layer protects the gastrointestinal epithelium against constant attack by digestive fluids, microorganisms, and toxins. A sulfur amino acid-deficient diet reduces intestinal mucosal growth associated with villus atrophy, reduces epithelial cell proliferation, lower goblet cell number and diminished small-intestinal redox capacity (Bauchart-Thevret C, 2009), whereas a dietary sulfur amino acid supplementation stimulates ileal mucosal growth after massive small bowel resection in rats (Shyntum *et al.*, 2009). Dietary supplementation of threonine, serine, cysteine, and proline stimulates mucin synthesis, influences the commensal microbiota, and restores gut barrier homeostasis in an animal model of ulcerative colitis (Arslan *et al.*, 2001; Jenkins *et al.*, 1988).

The intestinal tract is also one of the largest lymphoid organs in the body, and consists of immune cells in organized gut-associated lymphoid tissues (Field *et al.*, 2002). Cystine and cysteine are not taken up by the same amino acid transporter in human cells (Bannai, 1984). Cysteine is not considered an essential amino acid in humans; nevertheless, lymphocytes lack an essential enzyme for the production of cysteine from methionine, turning cysteine into an essential amino acid for lymphocytes (Eagle *et al.*, 1966). Moreover, T lymphocytes are unable to take up cystine, the main form of cysteine in the extracellular space. Therefore, the proliferation of T lymphocytes depends on antigen-presenting cells such as dendritic cells and macrophages that increase extracellular cysteine levels after antigen recognition, allowing the proliferation of T lymphocytes for the specific immune response (Angelini *et al.*, 2002; Gmunder *et al.*, 1990; Iwata *et al.*, 1994).

The final products of methionine and cysteine metabolism in eucaryotic metabolism are glutathione, homocysteine and taurine, which play important roles in the intestinal immune response (Grimble, 2006). Glutathione depletion

aggravates ileal inflammation after infection by *Salmonella enterica* serovar Enteritidis, but it does not play a role in intestinal permeability of *Salmonella*; however, cystine supplementation reduces intestinal permeability and *Salmonella* translocation, enhancing the gut barrier function by a mechanism unlikely to be related to glutathione (van Ampting *et al.*, 2009).

AIMS

The aims of this Thesis work are as follows:

- To study whether bile salts, which are well known DNA damaging agents, cause curing of the *Salmonella* virulence plasmid.
- To study whether the *ccdAB* genes of the *Salmonella* virulence plasmid, which encode a putative addiction module, contribute to plasmid stability.
- To investigate whether conjugal transfer of the *Salmonella* virulence plasmid occurs during animal infection.
- To identify the section of the murine intestine that supports conjugal transfer of the *Salmonella* virulence plasmid.
- To study whether FinP RNA has additional targets besides its cognate messenger RNA, *traJ.*
- To investigate whether increased *cysDNC* expression in the presence of FinP RNA affects conjugal transfer.
- To study whether L-cysteine and other sulfur-containing compounds inhibit conjugation.
- To compare the capacity of the virulence plasmids of three model *Salmonella* strains (LT2, ATCC 14028 and SL1344) to boost systemic infection and to promote conjugation.

RESULTS

Chapter 1: Bile-induced curing of the virulence plasmid in *Salmonella enterica* serovar Typhimurium

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Bile-Induced Curing of the Virulence Plasmid in *Salmonella enterica* Serovar Typhimurium[▽]

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Exposure to bile induces curing of the virulence plasmid in *Salmonella enterica* serovar Typhimurium (pSLT). Disruption of the *ccdB* gene increases pSLT curing, both spontaneous and induced by bile, suggesting that the pSLT *ccdAB* genes may encode a homolog of the CcdAB addiction module previously described in the F sex factor. Unlike the F sex factor, synthesis of pSLT-encoded pili does not confer bile sensitivity. These observations may provide insights into the evolution of virulence plasmids in *Salmonella* subspecies I, as well as the causes of virulence plasmid loss in other *Salmonella* subspecies.

Certain *Salmonella* serovars belonging to subspecies I carry a large plasmid of 50 to 90 kb (19). All *Salmonella* virulence plasmids share a 7. 8-kb region, *spv*, required for bacterial proliferation in the reticuloendothelial system (10). Other loci of the plasmid, such as the fimbrial operon *pef*, the conjugal transfer gene *traT*, and the *rck* and *rsk* genes may play roles in other stages of the infection process (19). The virulence plasmid of *Salmonella enterica* serovar Typhimurium (henceforth, pSLT) is self-transmissible (1); virulence plasmids from other serovars, such as *Salmonella enterica* serovars Enteritidis and Choleraesuis, carry incomplete *tra* operons (19). The presence of virulence plasmids in host-adapted serovars has suggested that virulence plasmid acquisition may have expanded the host range of *Salmonella*. However, *Salmonella* subspecies II, IIIa, IV, and VII do not contain a virulence plasmid and carry the *spv* region on the chromosome (4).

During animal infection, *Salmonella* is exposed to bile salts, which have at least two distinct antibacterial activities, as detergents that disrupt the cell envelope (11) and as DNA-damaging agents that cause DNA rearrangements and point mutations (17). Current evidence suggests that the primary DNA lesions caused by bile salts may involve oxidative damage (18). The bile concentrations encountered by *Salmonella* during the intestinal stage of infection are low and changing (13). However, systemic infection leads to colonization of the hepatobiliary tract, where the concentration of bile is high and steady (13). Furthermore, *Salmonella* can cause chronic infections: for instance, about 3% of humans surviving typhoid fever are chronic, asymptomatic carriers of *S. enterica* serovar Typhi (14), which usually resides in the gall bladder (9). *Salmonella* survival in the presence of bile salts requires a variety of defense functions, including envelope barriers and efflux pumps (11), as well as DNA repair functions able to cope with bile-induced DNA injuries (18).

Because DNA lesions can impair DNA replication, many DNA-damaging agents cause plasmid curing (24). Furthermore, the repertoire of DNA repair functions required for bile resistance suggests that bile salts may impair DNA replication in *S. enterica* (18). On these grounds, we considered the possibility that exposure of *Salmonella* to bile could cure the virulence plasmid. Below we show that bile is a curing agent indeed. We also show that the *ccdB* gene plays a role in virulence plasmid stability. Finally, we describe an unsuspected difference between pSLT and the F sex factor: while synthesis of F pili greatly increases sensitivity to bile salts (3), derepression of the pSLT *tra* operon does not cause bile sensitivity.

Exposure to bile causes virulence plasmid curing. Despite its low copy number (6), spontaneous loss of pSLT has not been reported in the literature, indicating that the plasmid is highly stable in *Salmonella* populations. To detect pSLT curing, we designed a positive selection strategy based on selecting tetracycline-sensitive derivatives of a tetracycline-resistant strain (15) (Table 1). For this purpose, a Tn*10* insertion (allele *zzv-6315*::Tn*10d*Tc) was introduced in pSLT, permitting the selection of Tc^s derivatives on Bochner-Maloy plates (15). To distinguish plasmid curing from other events causing tetracycline sensitivity (e.g., point mutations and deletions), a kanamycin resistance marker was also introduced in pSLT. The resulting virulence plasmid was thus tagged with two resistance markers, Tc^r and Km^r, both located in the *spv* region and separated by 7 kb, approximately (data not shown).

To obtain pSLT-cured derivatives, aliquots of saturated LB-grown cultures of strain LT2 were spread on Bochner-Maloy plates. Tc^s colonies were then replica printed to LB plates supplemented with kanamycin. Plasmid curing frequency was calculated as the ratio between the number of Km^s Tc^s isolates and the number of bacterial cells plated (determined by plate counts on LB agar). Most, if not all, Km^s Tc^s isolates obtained by this procedure were plasmidless, as indicated by their inability to receive a third, unlinked plasmid marker, *samA*::Cm^r: 19 of 19 independent Km^s Tc^s derivatives gave no Cm^r transductants when tested for receipt of *samA*::Cm^r by P22 HT-mediated transduction (20).

Spontaneous curing of the virulence plasmid occurred at frequencies below 10^{-6} (Fig. 1). The effect of bile on virulence

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TABLE 1. Genotypes of the bacterial strains used in this study

Strain	Genotype
LT2	Wild type
SV3000[a]	*dam-201*::Tn*10d*Tc
SV3003[a]	*traB*::Mu*dJ*
SV3081[b]	pSLT$^-$
SV4478[c]	*finO*::Km
SV4492[d]	*spvA*::Mu*dJ zzv-6315*::Tn*10d*Tc
SV4987	*spvA*::Mu*dJ zzv-6315*::Tn*10d*Tc Δ*ccdB*::Cm
SV5226	*spvA*::Mu*dJ zzv-6315*::Tn*10d*Tc Δ*ccdB*::Cm Δ*finO*
SV5228	*spvA*::Mu*dJ zzv-6315*::Tn*10d*Tc Δ*ccdB*::Cm *traB*::*MudJ*

[a] Strain described in reference 22.
[b] Strain described in reference 23.
[c] Strain described in reference 5.
[d] *spvA*::Mu*dJ* allele described in reference 12.

TABLE 2. MIC of sodium deoxycholate in strain LT2 and mutant derivatives[a]

Strain	Genotype	MIC (g/100 ml)
LT2	Wild type	5.3 ± 1.1
SV3000[b]	*dam-201*::Tn*10d*Tc	0.4 ± 0.1
SV3081[c]	pSLT$^-$	4.6 ± 1.4
SV3003	*traB*::Mu*dJ*	5.2 ± 0.8
SV4478	*finO*::Km	5.5 ± 1.0

[a] Exponential-phase cultures in LB broth were prepared. Samples containing around 3×10^2 CFU were transferred to polypropylene microtiter plates (Soria Genlab, Valdemoro, Spain) containing known amounts of sodium deoxycholate (Sigma Chemical Co, St. Louis, MO). After 12 h of incubation at 37°C, growth was visually monitored. Data are averages and standard errors of six independent experiments.
[b] Strain described in reference 22.
[c] Strain described in reference 23.

plasmid curing was tested by growing *S. enterica* in liquid LB containing different concentrations of ox bile extract. Aliquots from saturated cultures grown in LB-bile were spread on Bochner-Maloy plates, and Tc^s colonies were replica printed, as described above, to LB-kanamycin. Exposure to bile increased the frequency of Km^s Tc^s isolates in a dose-dependent fashion (Fig. 1), providing evidence that bile is a plasmid-curing agent.

Effect of *ccdB* disruption on virulence plasmid stability. The *Salmonella* virulence plasmid belongs to the F-like family, and contains DNA regions homologous to the F sex factor (19). One such region is *ccdAB*, which in F encodes an addiction module involved in plasmid stability (8). To investigate whether the *ccdAB* region of pSLT encoded a functional addiction module, a Ccd$^-$ mutant of *S. enterica* was constructed by gene targeting. The gene chosen for disruption was *ccdB*, which in F encodes the toxin of the addiction module (2). Disruption of *ccdB* was achieved by the procedure of Datsenko and Wanner (7), using the oligonucleotides 5′ CGGATCGTT TGCTGACGACAACAGGAACTGGTGATATGCAGTGT AGGCTGGAGCTGCTTC 3′ and 5′ CTGTTCGCTGACAC GCATATCAGATCCCCCGGAACATCAGCATATGAATA TCCTCCTTAG 3′. Two additional, external PCR primers were used to verify the predicted deletion: 5′ TGAGGTGGC CAGCTTTATAG 3′ and 5′ CAGAAACTCCGCACAC AGCC 3′. Primer design was based on the published genome sequence of the LT2 strain (16).

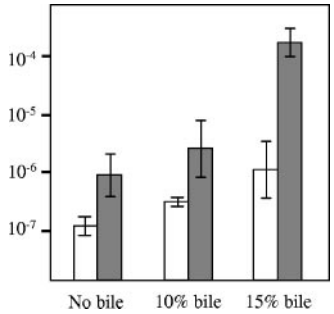

FIG. 1. Frequencies of curing of the *Salmonella* virulence plasmid in a Ccd$^+$ strain (white histograms) and in a Ccd$^-$ mutant (dark histograms). The strains used were the isogenic pair SV4492 (Ccd$^+$) and SV4987 (Ccd$^-$). Ox bile extract (sodium choleate) was purchased from Sigma Chemical Co., St. Louis, MO, and used as described elsewhere (17). Data for the Ccd$^+$ strain are averages of four independent experiments. Data for the Ccd$^-$ strain are averages of six independent experiments. Bars represent standard errors.

Trials of curing in a CcdB$^-$ pSLT plasmid were carried out as described above. The spontaneous frequency of pSLT curing increased 1 order of magnitude in a Ccd$^-$ background (Fig. 1), indicating that the *ccdAB* genes may encode a functional addiction module that contributes to pSLT stability. Curing of the CcdB$^-$ plasmid was strongly affected by bile and reached frequencies around or above 10^{-4} (3 orders of magnitude higher than the spontaneous frequency of curing in wild-type pSLT) in the presence of 15% ox bile extract (Fig. 1).

Virulence plasmid functions do not affect bile resistance. To investigate whether the presence of the virulence plasmid affected *S. enterica* survival in the presence of bile, we compared the MICs of sodium deoxycholate (DOC) in the wild type and in a pSLT-cured derivative. Aliquots from exponential cultures in LB broth, each containing around 3×10^2 colony-forming units, were transferred to polypropylene microtiter plates containing known amounts of DOC. After 12 h of incubation at 37°C, growth was visually monitored. As a control, a DNA adenine methylase (Dam$^-$) mutant was included in these experiments; *S. enterica* Dam$^-$ mutants are extremely sensitive to bile salts (17). Data shown in Table 2 indicate that curing of the virulence plasmid does not alter sensitivity of *S. enterica* to DOC. However, this observation left open the possibility that virulence plasmid functions which are usually repressed might alter bile sensitivity upon derepression. In fact, the *tra* operon of the F episome is known to sensitize *Escherichia coli* to bile salts (3). In F, bile salt sensitivity is caused by the *tra*-encoded type IV secretion system and requires an active F pilus assembly pathway (3). Unlike F, the *tra* operon of the *Salmonella* virulence plasmid is tightly repressed by the FinOP system (5, 21); hence, we considered the possibility that derepression of *tra* might confer bile sensitivity to *S. enterica.* Actually, *tra* operon derepression has been shown to cause bile sensitivity in another F relative, plasmid R100 (3). However, an *S. enterica* strain carrying a *tra* operon derepressed by a *finO* mutation did not show increased sensitivity to sodium deoxycholate (Table 2). In turn, a *traB* mutation, which prevents synthesis of pili, did not alter the MIC of DOC. A complementary observation was that neither a *finO* mutation nor a *traB* mutation had any effect on pSLT curing (Fig. 2).

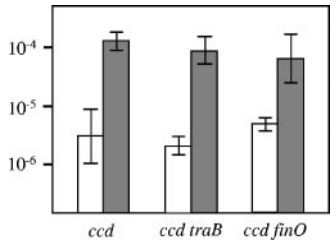

FIG. 2. Frequencies of curing of the *Salmonella* virulence plasmid in strains with different levels of *tra* operon expression. To increase the sensitivity of the assay, killing of plasmid-cured cells by CcdAB was avoided using a Ccd^- background. White histograms show spontaneous curing frequencies; dark histograms show curing frequencies in the presence of 15% ox bile extract. The strains used were the isogenic trio SV4987 ($CcdB^-$), SV5226 ($CcdB^-$ $FinO^-$), and SV5228 ($CcdB^-$ $TraB^-$). Data are averages of six independent experiments. Bars indicate standard errors.

Potential roles of bile in the evolution of salmonellae. A study on the distribution of *spv* genes among *Salmonella* subspecies considered that virulence plasmid instability might have favored *spv* translocation to the chromosome (and concomitant virulence plasmid loss) during the evolution of subspecies II, IIIa, IV, and VII (4). In this study, we suggest that bile could be a factor contributing to virulence plasmid instability in the ancestors of these subspecies. Bile concentrations of 15%, which induce significant rates of virulence plasmid curing under laboratory conditions, are commonly found in the gall bladder of humans and other mammals (13). Hence, bile can be predicted to impair the stability of the virulence plasmid in natural populations of *Salmonella* during systemic and chronic infections. Because bile can also induce DNA rearrangements (17), an attractive hypothesis is that both *spv* translocation and virulence plasmid loss could be caused by bile.

An additional, intriguing observation was that, unlike F and other F-like plasmids (3), synthesis of pSLT-encoded pili does not sensitize the host cell to bile salts. It is also noteworthy that certain pSLT-encoded Tra proteins show high divergence from their F counterparts, despite the overall conservation of *tra* operon organization and regulation in both plasmids. For instance, amino acid identities are only 68% for TraV, 33% for TraP, 29% for TraY, and 14% for TraS (data not shown). These observations may provide evidence that the type IV secretion apparatus encoded on the virulence plasmid has become bile resistant during the evolution of *Salmonella* subspecies I.

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Chapter 2: Conjugal transfer of the *Salmonella enterica* virulence plasmid in the mouse intestine

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Conjugal Transfer of the *Salmonella enterica* Virulence Plasmid in the Mouse Intestine▽

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BALB/c mice were infected with two *Salmonella enterica* serovar Typhimurium strains, one of which lacked the virulence plasmid. Transconjugants were found at high frequencies in the mouse feces and at low frequencies in the liver and the spleen, suggesting that mating occurred in the gut. Laboratory conditions that mimic those of the small intestine (microaerophilic growth in the presence of 0.3 M NaCl) increased the frequency of virulence plasmid transfer. Sodium deoxycholate, which is found at high concentrations in the duodenum, and sodium propionate, which is abundant in the large intestine, reduced the conjugation frequency. Feces inhibited conjugation. Altogether, these observations suggested that transfer of the virulence plasmid occurred in the distal portion of the small intestine. Conjugation trials in ileal loops provided direct evidence that conjugal transfer of the *Salmonella* virulence plasmid occurs in the ileum in mice.

Six decades after the discovery of bacterial conjugation, the underlying genetic and biochemical mechanisms are known in great detail, especially in model plasmids like the F sex factor and its relatives (15, 16), the Ti plasmid of *Agrobacterium tumefaciens* (47), the IncP-1 group of broad-host-range plasmids (2), R388 (19), R1162 (30), and pIP501 (1). In contrast, we have a more limited understanding of the environmental and physiological factors that affect conjugal transfer and of the natural habitats where conjugation occurs. Exceptions are transfer of *A. tumefaciens* Ti plasmids to plant cells in response to compounds present in the rhizosphere (47), pheromone-triggered mating in *Enterococcus faecalis* (20), and transfer of *Yersinia* plasmids in response to quorum sensing (4).

Animals harbor numerous bacterial species, which can establish a wide and complex range of interactions with their hosts. The possibility that plasmid transfer can occur inside animals has been considered since the 1960s, usually as a potential scenario for antibiotic resistance dissemination. Pioneering studies showed that transfer of drug resistance determinants in *Escherichia*, *Shigella*, and *Klebsiella* could be detected in the mouse intestine (26). A later, relevant observation was that drug resistance plasmids of *Neisseria* promoted conjugal transfer in mice even in the absence of antibiotic selection, suggesting that conjugation inside animals is not a rare or exceptional event (33). A study on conjugal transfer of plasmid R1*drd19* in mice provided evidence that *Escherichia coli* matings took place mainly in the mucus layer that covers the intestinal epithelium (27). In turn, another study showed that bacterial mating promoted by RP4 and F could take place inside eukaryotic cells (14). These few examples illustrate that animal organs can provide the appropriate environment for bacterial conjugation.

Salmonella enterica is an enteric bacterium that causes gastroenteritis and systemic infections in humans and other animals (29). In *S. enterica* subspecies I, a subset of virulence genes are located in an F-like plasmid, the so-called "*Salmonella* virulence plasmid" (34). All *Salmonella* virulence plasmids share a 7.8-kb region, *spv*, required for bacterial proliferation in the reticuloendothelial system (22). Other loci of the plasmid, such as the fimbrial operon *pef*, the conjugal transfer gene *traT*, and the *rck* and *rsk* genes, may play roles in other stages of infection but do not contribute to virulence in the standard mouse infection model (34).

The virulence plasmid of *S. enterica* serovar Typhimurium (usually known as pSLT) is self-transmissible (3, 7). As in other F-like plasmids, the pSLT genes necessary for conjugal transfer are clustered in an ~34-kb region (28). A single transcriptional unit (the *tra* operon) includes homologs of the F factor genes involved in pilus synthesis and assembly, surface exclusion, stabilization of mating aggregates, DNA nicking, DNA strand displacement, and DNA transport and the regulatory gene *finO* (28). As in F, transcription of the pSLT *tra* operon requires activation by the TraJ transcription factor, encoded by a nearby gene (7). In turn, *traJ* expression is under the control of the FinOP system of fertility inhibition, which in pSLT is fully functional and keeps conjugation tightly repressed (7, 37, 43). The *finP* gene is located in the antisense DNA strand of *traJ* and overlaps the 5′ region of *traJ* (10). The product of *finP* is an antisense RNA that acts together with the RNA chaperone FinO to prevent *traJ* mRNA translation and to promote *traJ* mRNA degradation (25, 45, 46).

Below, we describe surveys of pSLT transfer in the common house mouse, *Mus musculus* L., the natural host of *S. enterica* serovar Typhimurium. We show that pSLT is transferred at high frequencies during animal infection and present evidence that pSLT-promoted mating occurs in the distal portion of the small intestine (the ileum). Virulence plasmid transfer in the ileum might compensate for plasmid loss during intestinal passage. This view is consistent with the fact that bile salts, which have plasmid-curing capacity (18), are found at high concentrations in the mammalian small intestine, especially in the duodenum (24).

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MATERIALS AND METHODS

Bacterial strains. The strains of *S. enterica* used in this study {SV5534 [*trg*::Mu*d*Q(Cm^r) pSLT$^-$], SV5535 [Δ*dam-230*/pSLT *finO*::Km^r], SV5536 [Δ*finO spvA*::Tn*5*(Km^r)], and SV5556 [*spvA*::Tn*5*(Km^r)]} belong to serovar Typhimurium and derive from the mouse-virulent strain ATCC 14028. The Δ*dam-230* allele was constructed by in-frame deletion of the *S. enterica dam* gene encoding DNA adenine methylase (31). The Δ*finO* and *finO*::Km^r alleles have been described elsewhere (7, 18). The *trg*::Mu*d*Q allele is neutral for *Salmonella* virulence and provides a marker for strain discrimination in experimental infections of mice (35). The *spvA*::Tn*5* allele was described by Hensel et al. (23).

Media and chemicals. Luria-Bertani (LB) broth was used as rich medium. Solid LB medium contained agar at a 1.5% final concentration. Microaerophilic growth was achieved in GasPak incubation jars (Becton Dickinson Biosciences, San Agustín de Guadalix, Spain). Deoxycholic acid (sodium salt) and sodium propionate were both from Sigma Chemical Co., St. Louis, MO. Antibiotics were used at final concentrations described previously (42).

Bacterial matings in LB plates. Cultures of the donor and the recipient were grown overnight in LB broth. Cells were harvested by centrifugation and washed with LB broth (supplemented with 4% sodium deoxycholate [DOC] or 2% sodium propionate when necessary). Aliquots of both strains (500 μl each) were sucked onto a 0.45-μm-pore-size membrane filter. The donor/recipient ratio was 1:1. The filters were placed on LB plates and incubated at 37°C for 4 h. In certain experiments, LB plates contained 4% DOC or 2% sodium propionate (see below). After mating took place, the mixtures were diluted in 10 mM $MgSO_4$ and spread on selective plates. As controls, 0.1 ml of the donor and the recipient cultures was also spread on selective plates. Conjugation frequencies were calculated per donor bacterium. Matings under the so-called "*Salmonella* pathogenicity island 1 (SPI-1)-inducing conditions" involved slightly different conditions. Donor and recipient strains were grown overnight in LB broth without shaking. The LB plates contained 0.3 M NaCl, and the membrane-bound mating mixtures were incubated for 4 h at 37°C in GasPak microaerophilic jars.

Bacterial matings in LB broth. Overnight cultures of the donor and the recipient were prepared in LB broth. Aliquots of 500 μl were mixed to obtain a donor/recipient ratio of 1:1. Each mixture was centrifuged for 2 min at 13,000 rpm, and the supernatant was discarded. The pellet was resuspended in 50 μl of either LB broth or LB broth supplemented with feces, which had been previously homogenized in LB broth to obtain a final concentration of 1 g/liter. Mating mixtures were incubated at 37°C for 4 h. Diluted and undiluted aliquots were then spread on selective plates.

Bacterial matings in mice. Feeding of mice was suspended 1 day before inoculation. The donor and recipient strains were grown overnight in LB broth at 37°C without shaking. Under these conditions, the cultures reached saturation in the morning and contained around 3×10^8 bacterial cells/ml. The cultures were pelleted and resuspended in an aqueous solution containing 0.4% lactose and 0.9% NaCl. Each mouse was orally inoculated with 25 μl of donor bacterial suspension and with the same amount of recipient suspension. Each suspension contained 1×10^9 CFU. Inoculation of the second suspension (usually the donor) was performed 30 min after inoculation of the first (usually the recipient). Mouse sacrifice, extraction of liver and spleen, harvest and lysis of eukaryotic cells, and recovery of bacterial cells followed the procedures described by Shea et al. (36).

Bacterial matings in ileal loops. BALB/c mice were starved for 24 h and anesthetized before surgery by intraperitoneal injection of 300 μl of 2.5% avertin. A stock of 100% avertin was prepared by mixing 10 g of 2,2,2-tribromoethyl alcohol with 10 ml of *tert*-amyl alcohol (Sigma-Aldrich Química SA, Tres Cantos, Spain). Before use, this 100% stock was diluted to 2.5% in isotonic saline with vigorous stirring until it was dissolved. Mouse surgery was performed as follows. After a small incision was made, the small bowel was exposed and a loop was formed by ligating the intestine with silk thread at the ileocecal junction and at a site 5 cm proximal to the cecum. Bacterial suspensions in phosphate-buffered saline were then injected through a 25-gauge needle. The bowel was returned to the abdomen, and the incision was stapled. The mice were kept alive for 3 h and then sacrificed. Bacteria were recovered from the ileal loops by washing and homogenizing the loops in 0.5 ml of saline. Viable bacterial CFU were enumerated by plating aliquots of a dilution series of the homogenate onto LB agar. Donor and transconjugant colonies were distinguished by plating a dilution series onto LB agar with the appropriate combination of antibiotics.

Statistical analysis. Student's *t* test was used to compare conjugation frequencies. *P* values of ≤0.05 were considered significant.

RESULTS

Experimental conditions for the detection of conjugation in mice. Initial trials for the detection of *S. enterica* transconjugants after infection of mice were carried out using strain SV5535 (Δ*dam-230*/pSLT *finO*::Km^r) as the donor and SV5534 (*trg*::Mu*d*Q pSLT$^-$) as the recipient. Both strains are attenuated (by a *dam* mutation and by lack of the virulence plasmid, respectively), while transconjugants can be expected to be virulent. Use of a FinO$^-$ pSLT derivative was intended to increase the chances of detecting conjugation. The mice were sacrificed 6 days after infection. Km^r Cm^r transconjugants were recovered from the liver and the spleen, indicating that conjugation inside mice had indeed occurred. However, we observed that donor cells (Km^r Cm^s) were rare or absent in organs, suggesting that conjugation had occurred at an infection stage prior to organ colonization. Detection of transconjugants in feces provided additional evidence that conjugation occurs in the gut (see below). Albeit informative, the above-mentioned experiments presented a caveat: because transconjugants are more virulent than donors, transconjugants might have a growth advantage inside the animal; as a consequence, conjugation frequencies could be overestimated. To sort out this potential flaw, we devised crosses in which the donor, the recipient, and the transconjugants were equally virulent. Also, because the transconjugants were more abundant in the mouse feces than in deep organs, we chose the feces as the standard material for the calculation of conjugation frequencies. Hence, in subsequent experiments, we employed the following materials and conditions. (i) We used a donor strain whose virulence plasmid carried a Tn*5* insertion in *spvA* (23). The plasmidless recipient carried a chromosomal *trg*::Mu*d*Q marker, which is neutral for virulence (35). Lack of *spv* attenuates the virulence of donors, recipients, and transconjugants 100- to 1,000-fold (data not shown). As mentioned above, each genotype could be easily identified on plates: donors were Km^r, recipients were Cm^r, and transconjugants were Km^r Cm^r. (ii) Oral inoculation with a mixture of the donor and the recipient was avoided, since control experiments indicated that conjugal transfer readily occurred in the mixture: if aliquots from the mixture were spread on selective plates, Cm^r Km^r transconjugants were detected. Hence, the two strains were inoculated separately (30-min difference) by the oral route.

Detection of transconjugants in mouse feces. BALB/c mice were inoculated with strains SV5536 (Δ*finO spvA*::Tn*5*) and SV5534 (*trg*::MudQ pSLT$^-$). As a control, we confirmed that Cm^r, Km^r, and Cm^r Km^r bacterial CFU were absent from mouse feces prior to inoculation. Feces from infected mice were collected at 24-h intervals, homogenized in saline, diluted, and spread on antibiotic agar plates for the detection of Cm^r Km^r transconjugants. Colonies formed by Km^r Cm^s donors were also counted. Data from five matings are shown in Fig. 1. The frequency of transconjugants increased from day 2 to day 4 and seemed to reach a plateau after that. The mice were sacrificed at day 8.

The conjugation frequencies detected in these experiments were surprisingly high, even for a FinO$^-$ pSLT derivative (around 10^{-2}). Note that under optimal laboratory conditions, pSLT FinO$^-$ transfer occurs at frequencies between 10^{-4} and 10^{-3} (7). The donor and the recipient strains have similar

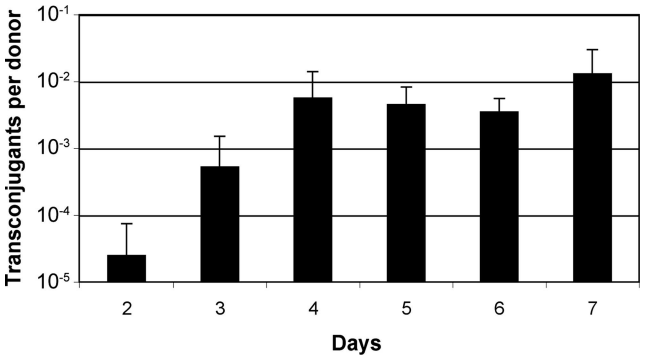

FIG. 1. Frequencies of transconjugants in the feces of four mice, measured at 24-h intervals, from day 2 to day 7 after inoculation. The donor was SV5536, and the recipient was SV5534. The error bars indicate standard deviations.

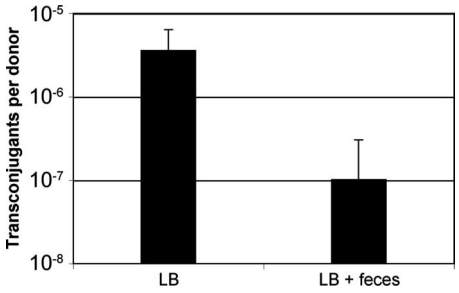

FIG. 2. Inhibition by feces of pSLT conjugal transfer. The conjugation frequencies in LB broth were compared with the corresponding frequencies in LB broth supplemented with feces. The donor was SV5536, and the recipient was SV5534. Shown are averages and standard deviations from seven independent matings.

growth rates under laboratory conditions, and they are equally virulent; hence, they can be expected to grow at similar rates inside the animal. It seems unlikely, therefore, that the frequencies found could be skewed by a growth advantage of the transconjugants. It is likely, however, that the frequencies found on later days were higher because conjugal transfer had increased the number of donors relative to the number of recipients.

Matings with a FinO$^+$ pSLT plasmid (strain SV5556) caused a delay in the detection of transconjugants, which started to appear at day 5 after inoculation, and the frequencies of transconjugants were lower than those obtained for pSLT FinO$^-$. Low frequencies made statistical analysis difficult, but a rough estimation was that the frequency of pSLT transfer decreased 100-fold in a FinO$^+$ background, a difference similar to that found between FinO$^+$ and FinO$^-$ pSLT under laboratory conditions (7).

A caveat for the above-mentioned experiments was that conjugation could occur not only inside the animal, but also in the feces. In other words, the total number of transconjugants might be the sum of those formed in the gut and those formed in the feces. To investigate this possibility, two kinds of experiments were carried out. (i) Feces collected on a given day were divided into aliquots. One aliquot was homogenized and immediately used to score transconjugants on selective plates. The other aliquots were kept at room temperature and used to score transconjugants after 24, 48, and 72 h. The numbers of transconjugants per donor did not increase over time (data not shown), suggesting that conjugation did not occur in the feces. (ii) Feces of uninfected mice were homogenized and added to LB broth at a final concentration of 1 g/liter. A 1:1 mixture of the donor and the recipient (SV5536 and SV5534, respectively) was added, and conjugation was allowed to proceed at 37°C for 4 h. Aliquots were spread on selective plates (LB broth supplemented with kanamycin and chloramphenicol) for the detection of transconjugants. Km^r Cm^s donors were enumerated by plating them on LB-kanamycin and replica printing to LB-chloramphenicol. Data from seven independent crosses are summarized in Fig. 2 compared with their corresponding controls (matings in LB broth). Lower frequencies of conjugal transfer in liquid cultures are typical of pSLT; for this reason, the standard protocol for pSLT transfer employs conjugation on membrane filters (7). Conjugation frequencies decreased 50-fold in the presence of feces, suggesting an inhibitory effect upon conjugation. Transfer of FinO$^+$ pSLT was likewise inhibited in the presence of feces: 5×10^{-7} in liquid LB broth versus 10^{-8} or less in liquid LB broth plus feces. We thus ruled out the possibility that conjugal transfer in feces might have skewed the calculation of conjugation frequencies inside mice. The evidence that feces strongly inhibit conjugation is discussed further below.

Effects of bile and propionate on pSLT transfer. The evidence that pSLT promoted *Salmonella* mating in the gut raised the question of which was the section of the gut where mating occurred. This issue was initially investigated using laboratory conditions that are known to mimic (obviously only to a certain extent) specific gut environments. (i) The proximal portion of the small intestine, especially the duodenum, contains high concentrations of bile (24). Previous studies in our laboratory had shown that, besides their detergent activities, bile salts are DNA-damaging agents that cause point mutations, gene rearrangements, and plasmid curing (18, 31, 32). To investigate whether bile salts affected pSLT-mediated conjugation, crosses were performed in the presence of DOC. The concentration used (4%) was lower than the DOC MIC in vitro (7%) and thus did not impair *S. enterica* viability. The donor strain was either SV5536 (FinO$^-$) or SV5556 (FinO$^+$). The recipient was SV5534 in all cases. Donor and recipient cultures were prepared and mixed (1:1) using the standard protocol for conjugation on a solid support (a membrane filter on top of agar). The presence of DOC caused an ~10-fold decrease in the frequency of pSLT transfer (Fig. 3), suggesting that conjugation is inhibited in the presence of bile. Despite the extreme reductionism of these experiments, a tentative conclusion is that conjugation is unlikely to occur in the duodenum. (ii) The large intestines of mammals contain metabolites that are rare or absent in the small intestine. For instance, high concentrations of propionate and butyrate are produced as a consequence of D-mannitol utilization by intestinal bacteria (50). We investigated whether the presence of 2% sodium propionate (a concentration that does not inhibit growth) affected the frequency of pSLT transfer under laboratory conditions. An ~10-fold decrease in the frequency of transconjugants was found for both wild-type pSLT and its FinO$^-$ derivative (Fig. 3). Albeit cautiously, we propose that inhibition of conjugal trans-

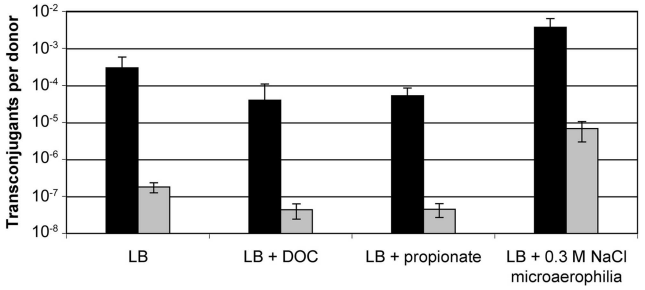

FIG. 3. Effects of DOC, sodium propionate, and SPI-1-inducing conditions on conjugal transfer of pSLT FinO$^-$ (black histograms) and pSLT FinO$^+$ (gray histograms). Matings were carried out on membrane filters. The donors were either SV5536 (FinO$^-$) or SV5556 (FinO$^+$), and the recipient was SV5534. The data are averages and standard deviations from six independent matings. Statistical analysis indicated that the conjugation frequencies in LB broth were significantly different from those in LB broth plus DOC, LB broth plus propionate, and LB broth plus 0.3 M NaCl. *P* values of 0.017, 0.022, and <0.0001, respectively, were obtained for crosses involving a FinO$^-$ donor. *P* values were all <0.0001 for crosses involving a FinO$^+$ donor.

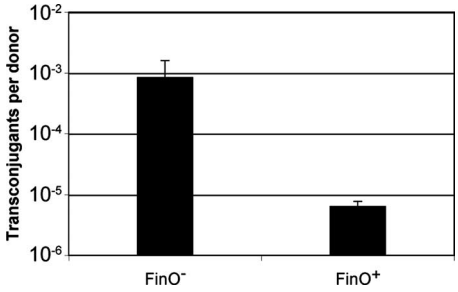

FIG. 4. Transfer of FinO$^+$ and FinO$^-$ pSLT plasmids in ileal loops of mice. The donors were SV5536 (FinO$^-$) and SV5556 (FinO$^+$), and the recipient was SV5534. The data are averages and standard deviations from three independent matings.

fer by propionate may indicate that mating inside mice does not occur in the large intestine. Propionate, which is likewise present in feces, may also contribute to inhibition of mating in the presence of feces (Fig. 2).

Effects of SPI-1-inducing conditions on pSLT transfer. If the results described above faithfully reproduce conditions found in specific sections of the mouse intestine, the hypothetical scene of conjugal transfer should be an intestinal section where the concentrations of bile and propionate are low. These conditions are met by the distal portion of the small intestine, especially the ileum. Additional traits of the ileum are microaerophilia and relatively high osmolarity (29). In fact, microaerophilic incubation in LB broth containing 0.3 M NaCl is a standard procedure to reproduce in vitro the conditions that induce expression of SPI-1 in the ilea of infected animals (13). On these grounds, we tested the effect of microaerophilic growth in the presence of 0.3 M NaCl on pSLT transfer. Matings were performed as described above, with the difference that the mating mixtures were incubated in a GasPak microaerophilic jar. An 8- to 10-fold increase in the number of transconjugants was found (Fig. 3), indicating that ileum-like laboratory conditions stimulate conjugal transfer. The high conjugation rates found (between 10^{-3} and 10^{-2} per donor cell for FinO$^-$ pSLT and nearly 10^{-5} for the wild-type virulence plasmid) suggest that pSLT transfer may be stimulated under the same conditions that trigger invasion of the intestinal epithelium by *Salmonella*. Interestingly, high rates of conjugal transfer in anaerobiosis have also been described for the *E. coli* F sex factor (38).

Conjugal transfer of pSLT in murine ileal loops. Because several lines of evidence suggested that pSLT transfer inside mice might occur in the distal portion of the small intestine, we devised conjugation trials in the ilea of BALB/c mice. Donor and recipient suspensions were prepared in phosphate-buffered saline to avoid injection of LB medium (which obviously supports conjugation) into the mouse intestine. Control matings indicated that both pSLT FinO$^+$ and pSLT FinO$^-$ were transferred at very low frequencies in phosphate-buffered saline: 10^{-5} and $<10^{-7}$ transconjugants per donor, respectively. Ligated ileal loops of murine intestine were inoculated with donor and recipient strains (10^8 CFU each). As described above, the donor and recipient bacterial suspensions were inoculated separately. Three crosses were performed with FinO$^+$ pSLT (strain SV5556) and another three with a FinO$^-$ derivative (SV5536). The recipient was SV5534 in all cases. Three hours after inoculation, the mice were sacrificed and bacterial cells were recovered from the ileal loops. Transconjugants and donors were enumerated on selective media. Transfer of the FinO$^-$ virulence plasmid was detected at frequencies around 10^{-3} (Fig. 4). As expected, the parental FinO$^+$ plasmid was transferred at frequencies 100-fold lower (Fig. 4). The fact that the conjugation frequencies in ileal loops were lower than those found in mice may simply reflect the shorter mating times allowed. Surgical manipulation and injection of the bacterial suspensions might also have perturbed the microaerophilic environment and reduced osmolarity. Whatever the case, these experiments provided direct evidence that the *Salmonella* virulence plasmid can be conjugally transferred in the ilea of infected mice.

DISCUSSION

Phylogenetic analysis suggests that *S. enterica* virulence plasmids are transferred at high rates in nature (6). However, the model *Salmonella* virulence plasmid (the pSLT plasmid of *S. enterica* serovar Typhimurium) carries a functional FinOP system of fertility inhibition and promotes conjugation at very low frequencies (3, 7, 37). This contradiction may have more than one resolution. There is the possibility that pSLT is a rare exception and that many natural *Salmonella* virulence plasmids are derepressed for transfer, like the *E. coli* F episome (15). However, it is also conceivable that transfer in nature may follow rules different from those observed in the laboratory. In support of the latter view, an intriguing feature of F-like plasmids is the fact that their transfer functions are under the control of an ample variety of host-encoded regulators. The current list includes the redox sensor ArcA (41), the global transcriptional regulators Lrp (7, 9, 40) and CRP (39), the nucleoid protein H-NS (10, 49), the RNA chaperone Hfq (48), the GroEL heat shock chaperone (51, 52), DNA adenine methylation (43), and the extracytoplasmic stress response CpxAR system (21). Certain regulators have slightly different

functions depending on the plasmid, suggesting that each plasmid may have adapted to the particular lifestyle of its host (8, 9). Even under laboratory conditions, different F-like plasmids seem to follow different transfer rules: for instance, F transfer is inhibited in stationary-phase cultures (17) while pSLT transfer is not (7). In turn, pSLT transfer occurs at lower frequencies in LB broth than in minimal medium, a trait not found in F (3). A tentative interpretation for the existence of so many controls is that F-like plasmids may promote mating in response to specific physiological and environmental signals and increase their conjugation rates in appropriate environments.

Conjugal transfer in the mouse intestine may be an example of activation of conjugal transfer under convenient circumstances. The gut provides a rich supply of nutrients, which permit rapid multiplication of *Salmonella* cells. However, certain sections of the gut may not be suitable for conjugation. For instance, conditions that mimic the duodenum (LB broth plus DOC) inhibit mating. Although synthesis of pSLT Tra pili does not cause bile sensitivity as it does in F (18), it is conceivable that cell-to-cell interactions can be disturbed by the presence of bile salts. Transfer of pSLT is also inhibited in LB broth plus propionate, suggesting that the large intestine may likewise be inappropriate to support conjugation. In fact, *Salmonella* is known to be at a disadvantage when it competes with the bacterial flora of the large intestine, apparently as a consequence of inefficient nutrient uptake (44). Because conjugation is an energetically expensive process (11), its inhibition may make full sense under such conditions.

Can our laboratory observations be extrapolated to nature? In principle, the BALB/c mouse can be expected to be a reliable model for the study of conjugation in the intestines of animals. Lack of the macrophage-associated protein N-RAMP renders BALB/c mice especially sensitive to bacterial infections; however, the effects of N-RAMP loss are much less pronounced at the intestinal stage of infection than during systemic infection (12). On the other hand, *Salmonella* colonization of animals requires competition with the established intestinal flora. For this reason, treatment of mice with antibiotics prior to *Salmonella* administration is an old practice used to boost experimental *Salmonella* infections (5). However, we detected high frequencies of conjugal transfer without antibiotic pretreatment of mice (in other words, under circumstances that do not especially favor *Salmonella* proliferation). Altogether, these considerations suggest that intestinal transfer of virulence plasmids may indeed occur in house mice, and perhaps in other animals, during *Salmonella* infection.

The detection of *Salmonella* conjugation in the mouse intestine does not exclude the possibility that other environments may also provide suitable conditions for virulence plasmid transfer. In fact, transfer of a close relative of pSLT, the F sex factor, has been detected inside cultured epithelial cells (14). However, the population sizes and cell densities attained by *Salmonella* in the small intestine (29) are unlikely to be found in other environments, either inside animals or outside. Transfer of the virulence plasmid in the intestines of animals might thus account for the high conjugation rates predicted by phylogenetic analysis (6).

ACKNOWLEDGMENTS

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Chapter 3: Regulation of conjugation by L-cysteine in *Salmonella enterica*

Regulation of conjugation by L-cysteine in *Salmonella enterica*

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ABSTRACT

FinP, an antisense RNA encoded on the *Salmonella enterica* virulence plasmid (pSLT) and other F-like plasmids, inhibits conjugal transfer by downregulating *traJ* mRNA. Because FinP RNA is abundant in the cell while *traJ* mRNA is scarce, we investigated whether FinP RNA could have additional regulatory functions. Transcriptomic and quantitative RT-PCR analyses showed that FinP RNA controls the expression of several chromosomal loci. One such locus is the *cysDNC* operon, whose products catalyze biosynthetic reactions collectively known as the sulfate reduction pathway for L-cysteine biosynthesis. Expression of *cysDNC* is enhanced in the presence of FinP RNA. *S. enterica* mutants lacking either ATP sylfurylase or NADPH-sulfite reductase underwent elevated rates of pSLT transfer, suggesting that compounds of the L-cysteine biosynthetic pathway might inhibit conjugation. In support of this hypothesis, exogenous L-cysteine and sodium sulfide were found to be inhibitors of pSLT transfer.

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INTRODUCTION

In F-like plasmids, expression of plasmid conjugation functions is severely restrained by the so-called fertility inhibition system (Firth *et al.*, 1996), whose existence was discovered in the 1970's (Finnegan & Willetts, 1972; Finnegan & Willetts, 1973). This system is composed of an antisense RNA, FinP, and an RNA chaperone, FinO, which act together to prevent expression of *traJ* mRNA and to stimulate its turnover (Ghetu *et al.*, 1999; Jerome *et al.*, 1999; Lee *et al.*, 1992; van Biesen *et al.*, 1993; van Biesen & Frost, 1994). The *traJ* gene encodes a transcription factor necessary for transcription of the main operon, *tra* (Firth *et al.*, 1996). The F sex factor is a *finO* mutant (Cheah & Skurray, 1986), a fortunate trait that facilitated the discovery of bacterial conjugation (Lederberg & Tatum, 1953) and revealed the existence of extrachromosomal ("episomic") DNA molecules (Hayes, 1953). However, other F-like plasmids contain a wild-type FinOP system that keeps *tra* operon expression at very low levels. One such plasmid is the virulence plasmid of *Salmonella enterica* serovar Typhimurium, also called pSLT (Rotger & Casadesus, 1999; Smith *et al.*, 1973; Spratt & Rowbury, 1973). Although F and R100 remain classical models in the study of F-like plasmids, pSLT presents unique features such as the possession of a gene cluster that contributes to systemic infection of animals (Gulig *et al.*, 1992) and the ability to promote conjugation in the intestine of infected animals (Garcia-Quintanilla *et al.*, 2008). Transfer of pSLT is tightly repressed in batch cultures of *S. enterica* (Camacho & Casadesus, 2002), and becomes derepressed in the ileum (Garcia-Quintanilla *et al.*, 2008). High osmolarity and microaerophilia, two conditions that reductionistically mimic intestinal conditions, derepress pSLT transfer under laboratory conditions (Garcia-Quintanilla *et al.*, 2008).

Transcription of the pSLT *traJ* gene is repressed by Dam methylation, and activated by the leucine-responsive regulatory protein, Lrp (Camacho & Casadesus, 2002). Dam methylation keeps *traJ* transcription at very low levels, probably by restricting Lrp binding to replication fork passage (Camacho & Casadesus, 2005). Repression by the nucleoid protein H-NS is an additional factor that contributes to pSLT *traJ* repression (Camacho *et al.*, 2005). In contrast, the overlapping *finP* gene is driven by a strong σ^{70}-dependent promoter, and its transcripts are extremely abundant (Camacho *et al.*, 2005). A crucial factor to achieve high transcription rates of *finP* is Dam methylation, which prevents repression of the *finP* promoter by the nucleoid protein H-NS (Camacho *et al.*, 2005).

In this study, we show that FinP RNA increases expression of several chromosomal loci, including the *cysDNC* operon. Use of donor strains that lacked the sulfate reduction pathway increased conjugal transfer of pSLT, unveiling a hitherto unkown link between L-cysteine biosynthesis and bacterial conjugation. In support of this hypothesis, exogenous L-cysteine and sodium sulfide were found to inhibit conjugal transfer of pSLT. Hence, FinP-mediated enhancement of *cysDNC* expression inhibits mating, presumably by increasing L-cysteine synthesis. Hence, the FinOP system of pSLT turns out to be more sophisticated than previously envisaged, since fertility inhibition involves not only downregulation of *traJ* mRNA (Ghetu *et al.*, 1999; Jerome *et al.*, 1999) but also FinP-mediated upregulation of a chromosomal locus (*cysDNC*) whose products contribute to mating inhibition.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, media and culture conditions. Strains of *Salmonella enterica* serovar Typhimurium (often abbreviated as *S. enterica*) used in this study are listed in Table 1. Plasmid pKP32-3, a pBAD18 derivative expressing the pSLT *finP* gene from the arabinose-dependent p_{BAD} promoter, is described below. Transductional crosses using phage P22 HT 105/1 *int201* [(Schmieger, 1972) and G. Roberts, unpublished] were used for strain construction operations involving chromosomal markers, and for transfer of plasmids among *Salmonella* strains. The transduction protocol was described elsewhere (Garzon *et al.*, 1995). To obtain phage-free isolates, transductants were purified by streaking on green plates. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Luria-Bertani (LB) broth was used as standard liquid medium. Except if indicated otherwise, the carbon source was 0.2% glucose. Solid LB contained agar at 1.5% final concentration. Green plates were prepared according to Chan et al. (Chan *et al.*, 1972), except that methyl blue (Sigma-Aldrich, St. Louis, MO) substituted for aniline blue. Antibiotics were used at the concentrations described previously. L-cysteine. L-methionine, L-threonine, and sodium sulfide were from Sigma Chemical Co.

Designation	Genotype	Reference or source
ATCC 14028	Wild type	(Fields *et al.*, 1986)
LT2	Wild type	SGSC*
SV4711	pKD46	Lab stock
SV4478	pSLT Δ*finO*::Km^r **	(Camacho & Casadesus, 2005)
SV5556	pSLT *spvA*::Tn5*d*Km	(Garcia-Quintanilla *et al.*, 2008)
SV5534	*trg*::mu*d*Q (Cm^r) pSLT$^-$	(Garcia-Quintanilla *et al.*, 2008)
SV5729	Δ*cysD* / pSLT *spvA*::Tn5*d*Km	This study
SV5795	Δ*cysI* Δ*asrC* / pSLT *spvA*::Tn5*d*Km	This study
SV5505	pKP32-3	This study
SV5507	*cysD*::3xFLAG	This study
SV5722	cysD::3xFLAG / pKP32-3	This study
SV5723	*cysN*::3xFLAG /pKP32-3	This study

Table 1. Strain list
* SGSC: *Salmonella* Genetic Stock Centre, University of Calgary, Alberta, Canada
** The background of this strain is LT2, the rest strains are ATCC 14028

Construction of plasmid pKP32-3. Plasmid pKP32-3, a pBAD derivative expressing *finP* from the arabinose-dependent p_{BAD} promoter, was constructed similar to pBAD-RybB (Papenfort *et al.*, 2006) but using primers 5' GGA CAC ATA GGA ACC TCC T 3' and 5' TTT TTC TAG ATA TAG TGT CTT TGT AGT T 3' for insert amplification.

Transcriptome analysis. Strain SL1344 was transformed with plasmids pKP8-35 (control) and pKP32-3 (p_{BAD}-*finP*), and grown in liquid culture from single colonies to an OD_{600} of 1.5. sRNA expression was induced with L-arabinose (0.2% final concentration) for 10 min. For sample preparation 3 OD aliquots were removed and treated with 0.2 volumes of stop solution (5% water-saturated phenol, 95% ethanol), and snap-frozen in liquid nitrogen. RNA was isolated as described before (Promega SV total RNA purification kit). The microarrays used in this study include PCR products of all the genes present in the sequenced *S. typhimurium* strain LT2. In addition, we added 229 genes specific to *S. typhimurium* strain SL1344. Details of

all the amplicons can be found at http://www.ifr.ac.uk/Safety/MolMicro/pubs.html. Our experimental design involves the use of *Salmonella enterica* serovar Typhimurium genomic DNA as the co-hybridized control for one channel on all microarrays. This method has the advantage of allowing the direct comparison of multiple samples. Total RNA and chromosomal DNA were labeled by random priming according to the protocols described at http://www.ifr.bbsrc.ac.uk/safety/microarrays/protocols.html. Briefly, 16 µg RNA were reverse transcribed and labelled with Cy3-conjugated dCTP (Pharmacia) using 200U of Stratascript (Stratagene) and random octamers (Invitrogen). Chromosomal DNA (400 ng) was labelled with Cy5-dCTP using the Klenow fragment. After labelling, each Cy3-labelled cDNA sample was combined with Cy5-labelled chromosomal DNA and hybridised to a microarray overnight at 65ºC. After hybridisation, slides were washed and scanned using a GenePix 4000A scanner (Axon Instruments). Fluorescent spots and the local background intensities were identified and quantified using Bluefuse software (BlueGnome, Oxford, UK). To compensate for unequal dye incorporation, data centring to zero was performed for each block (one block being defined as the group of spots printed by the same pin). We considered genes to be differentially expressed if they displayed ≥ 3-fold changes in all replicates and were statistically significantly different (FDR ≤ 0.05) using Significance Analysis of Microarrays (Tusher *et al.*, 2001). Data visualization and data mining was performed using GeneSpring 7.3 (Agilent).

Construction of strains carrying *cysD, cysI,* and *asrC* deletions in the *Salmonella* chromosome. Deletions of *cysI* and *asrC* were generated by the method of Datsenko and Wanner (Datsenko & Wanner, 2000). Deletion of *cysD* was generated in frame by method of Ellermeier et al. (Ellermeier *et al.*, 2002), in order not to affect *cysN* and *cysC* expression downstreams. The kanamycin resistance cassette introduced during construction was excised by recombination with plasmid pCP20 (Datsenko & Wanner, 2000). Primers for *cysD* deletion were designed to eliminate 645 bp in the *cysD* coding sequence (from position +305 to position +949). The sequences of these oligonucleotides were 5' TCG CGA TCG TAC CGC CAA CGC ATA CGG CTG CGA ATT GCT GAT TCC GGG GAT CCG TCG ACC 3', and 5' TGG CGA TTT GTT GTG CAA GTA TGG TGT TCA TGG CGG CTC CGT GTA GGC TGG AGC TGC TTC 3'. The resulting deletion eliminates the entire *cysD* gene except 264 bp at its 5' end. PCR amplification using primers from both sides of the *cysD* locus identified kanamycin-sensitive isolates that carried the desired deletion. The sequences of these primers were 5' GAT GCG CCA TAA TGA TGA TC 3' and 5'

GTC ATC AAC GCT GCC ACA GG 3'. A 1704 bp deletion in the *cysI* ORF was generated with primers 5' GCG TAG AGC GCC GTT ATC AGC GAG ATG TCT ACT AAT GAG CGT GTA GGC TGG AGC TGC TTC 3', and 5' TTA TCA GGC CTA CAC AGA CGC CAT CCG GCA ATT CAG GTT ACA TAT GAA TAT CCT CCT TAG 3', and verified with primers 5' GAT CAA TGA TGG CGC GCA TA 3', and 5' AGA GCC ATT ACG CGA TCG AC 3'. A 1002 bp deletion in the *asrC* ORF was created with primers 5' GCG CAA CGT TTT GCC GAT TAA GGA GAA CAT CAT GAG CAT TGT GTA GGC TGG AGC TGC TTC 3', and 5' GCA GCC TGT GGG CGG GCT GCG GCC ATG GCG TGG TTG ATT ACA TAT GAA TAT CCT CCT TAG 3'. Primers for *asrC* deletion verification by PCR amplification were 5' TAT GCA GGC CAT CGT CGT TG 3', and 5' ACC TCG CTA ACG ATT ACC TC 3'.

Tagging of the CysD, CysN and CysC proteins with a 3xFLAG epitope. Addition of a 3xFLAG epitope tag at the 3' end of the *cysD* gene was carried out using plasmid pSUB11 (Km^r, 3xFLAG) as template (Uzzau *et al.*, 2001). A *S. enterica* chromosomal fragment containing the appropriate region of the *cysD* gene was PCR-amplified using primers 5' GGC GGG GTC GAT GGA GCT TAA AAA ACG TCA GGG GTA TTT CGA CTA CAA AGA CCA TGA CGG 3', and 5' TGG CGA TTT GTT GTG CAA GTA TGG TGT TCA TGG CGG CTC CCA TAT GAA TAT CCT CCT TAG 3'. The resulting PCR fragment was purified, and used to electroporate *S. enterica* SV4711. Transformants were selected on LB-kanamycin. Incorporation of the 3xFLAG tag was proven by PCR amplification and DNA sequencing. The primers used for this amplification were 5' GAT GCG CCA TAA TGA TGA TC 3' and 5' GTC ATC AAC GCT GCC ACA GG 3'. Construction of CysN-3xFLAG and CysC-3xFLAG proteins was achieved by the same procedure. Primers for CysN-3xFLAG construction were 5' GGA CGC CCG AGA TTT GCT GGG AGA TAA ACA TGG CGC TGC AGA CTA CAA AGA CCA TGA CGG 3', and 5' GGC GAC AGT AAC GGG ATG AGA GTG CCA GAC CAC GTT CTC ACA TAT GAA TAT CCT CCT TA 3'. Primers for CysN-3xFLAG verification were 5' ACC TGA CCT TTG CTG AAC AG 3', and 5' TGA AGG CGT CTG ACC TTG TG 3'. CysC-3xFLAG was constructed with primers 5' ATT ATT AGA CCT GCT GAG ACG GCG CGA TAT TAT CAG ATC CGA CTA CAA AGA CCA TGA CGG 3', and 5' GTA ACC TTG ACC ATA CCG GGC AT CAA GCC CGG TGG TGT CCA TAT GAA TAT CCT CCT TAG 3', and verified with primers 5' GAA GAG GCG TTA CAT CAG CG 3' and 5' TGA AGG CGT CTG ACC TTG TG 3'.

Northern hybridization. RNA was prepared by the acid-hot-phenol method from stationary cultures in LB medium. RNA concentrations were estimated from OD_{260}

values. For Northern analysis, 7.5 mg of total RNA were separated under denaturating conditions either in 8 M urea- 8% acrylamide in TBE (Tris-Borate-EDTA pH 8.3) buffer or in formaldehyde (2.2 M)-agarose (1.3%) gel electrophoresis in MOPS ([N-morpholino] propanesulfonic acid-Sodium Acetate-EDTA pH7.0) buffer. For acrylamide gels, transfer of the RNA onto Hybond-N^+ membrane (Amersham), was performed with a semidry electrotransfer apparatus (Transblot SD; BioRad); in the case of agarose gels, transfer to the same support was done using a vacuum blotter after mild (50 mM NaOH, 10 mM NaCl) denaturation treatment. RNA was crosslinked to the membrane by UV irradiation using a Stratagene UV Stratalinker 2400. Membranes were hybridized to probes as follows: 5 pmol of oligonucleotide (Table 4) were 5' end-labeled using 10 U of T4 polynucleotide kinase (New England Biolabs) and 30 mCi of [-32P] ATP (3,000 mCi mmol-1, Amersham). Unincorporated radioactivity was eliminated by passage through Micro-Bio Spin 6 chromatography columns (BioRad). Hybridization was carried out in Ambion Oligonucleotide Hybridization Buffer at 45-50°C following Ambion's protocol. RNA was analysed with the ImageQuant software package. The *finP* probe used was the 20-mer 5' TAA TCG CCG ATA CAG GGA G 3', as previously described (Camacho *et al.*, 2005). The *traJ* probe was 5' TGC TCT AGA ACA CAT AGG AAC CTC CTC 3'. The *ompA* probe was 5' GAG CAA CCT GGA TCC GAA AG 3'.

Quantitative reverse transcriptase PCR and calculation of relative mRNA expression levels. RNA was extracted from stationary phase cultures using the SV total RNA isolation system (Promega Corporation, Madison, Wisconsin). The quantity and quality of the extracted RNA were determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). To reduce genomic DNA contamination, the preparation was treated with DNase I (Turbo DNA free, Applied Biosystems/Ambion, Austin, Texas). An aliquot of 0.5 µg of DNase I-treated RNA was used for cDNA synthesis using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California). Real-time PCR reactions were performed in an Applied Biosystems 7500 Fast Real-time PCR System. Each reaction was carried out in a total volume of 15 µl on a 96-well optical reaction plate (Applied Biosystems) containing 7.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems), 6.9 µl cDNA (1/10 dilution), and two gene-specific primers at a final concentration of 0.2 µM each. Real-time cycling conditions were as follows: (i) 95°C for 10 min; (ii) 40 cycles at 95°C for 15 s, 60°C for 1 min. No-template and no reverse-transcriptase controls were included for each primer set and template. Melting curve analysis verified that each reaction contained a single PCR product.

Reported gene expression levels were normalized to transcripts of *rfaH*, which served as an internal standard. Gene-specific primers, designed with PRIMER3 software (http://primer3.sourceforge.net), were as follows: for *rfaH* were described elsewhere (Sittka *et al.*, 2008); for *finP,* 5' GGA CAC ATA GGA ACC TCC TCA A 3', and 5' TGT CAC TCC CTG CAT CGA CT 3'; for *traJ*, 5' TCA GCC TCT TTC GGG AGA TAG T 3', and 5' AGC GAC TGA CAT TCA AGT TCC A 3'; for *cysD,* 5' ATT ATC CGT GAA GTG GCA GCG 3', and 5' ACC CGG ATA AAA CGC CTT ACG 3'; for *cysN*, 5' GTT TGC TGC ACG ATA CTC TGC 3', and 5' ATC TAC CAG CAG CGC CAG ATC 3'; and for *cysC*, 5' GTT CGG GAA AAT CAA CGG TGG 3' and 5' GTG GCG CAC ATT ATC ACC ATC 3'. Student's *t* test was used to analyze results. *P* values of 0.05 or less were considered significant.

Matings. Cultures of the donor and the recipient were grown overnight in LB broth. Cells were harvested by centrifugation and washed with LB. Aliquots of both strains, 500 µl each, were sucked onto a membrane filter 0.45 mm pore size. The donor/recipient ratio was 1:1. The filters were placed on LB plates, and incubated during 4 h at 37ºC in GasPak microaerophilic jars (Camacho & Casadesus, 2002). Supplements were added at the concentrations indicated in the corresponding sections. Conjugation frequencies were calculated per donor cell, as previously described (Camacho & Casadesus, 2002; Garcia-Quintanilla *et al.*, 2008).

RESULTS

Expression levels of the *S. enterica finP* and *traJ* genes. To monitor the levels of *finP* and *traJ* transcripts encoded on pSLT, two kinds of experiments were performed:

(i) Northern analysis using RNA preparations from isogenic strains carrying *finO*$^+$ pSLT and an isogenic *finO* derivative (Fig. 1, panel A). Hybridization against *finP* and *traJ* probes revealed relatively high amounts of FinP RNA, even in the absence of FinO. In contrast, the *traJ* transcript was hardly visible, not even in the RNA preparation containing *finO* pSLT (Fig. 1, panel A).

(ii) Quantitative reverse transcriptase PCR experiments were performed to compare the amounts of FinP RNA and *traJ* mRNA. Primer pairs for the reactions were as

described in Materials and Methods. FinP RNA was found to be >100 more abundant than *traJ* mRNA (Fig. 1, panel B).

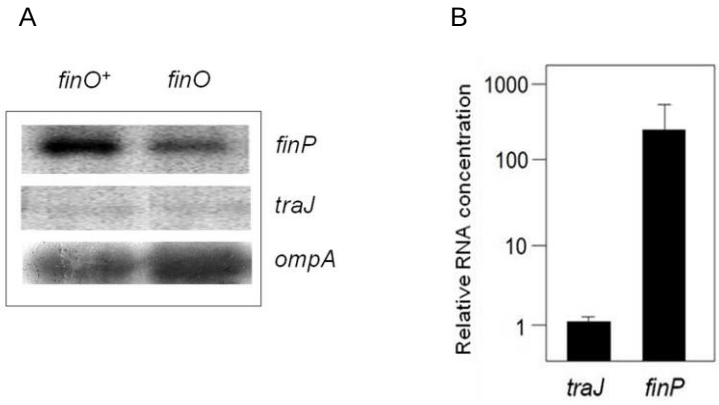

Figure 1. A. Levels of FinP RNA and *traJ* mRNA in *finO*⁺ and *finO* isogenic strains of *S. enterica* (LT26 and SV4478, respectively). As a control, *ompA* mRNA was used. **B.** Relative amounts of *traJ* mRNA and FinP RNA, normalized to *rfaH* mRNA, in LB medium at early stationary fase (~1.5). Each bar represents the average from three independent experiments.

Identification of FinP target RNAs by microarray analysis. The existence of excess FinP RNA in pSLT-containing *Salmonella* prompted a search for additional FinP targets among *S. enterica* transcripts, either plasmid-encoded or chromosomal, using high throughput analysis. Induction of *finP* expression from the arabinose-dependent promoter of pKP32-3 was achieved with 0.2% arabinose, and changes in gene expression patterns under such conditions were detected. Only RNAs which showed altered after statistical filtering were taken into account. Higher mRNA content upon FinP overproduction provided evidence for activation of gene expression mediated by FinP. In turn, lower mRNA content upon FinP overproduction provided evidence for inhibition of gene expression by FinP RNA. Especially relevant data are summarized in Table 2. The existence of altered mRNA levels was confirmed by quantitative RT-PCR for the *cysD* transcript (see below), and for the *ygaE* and *STM4302* transcripts as well (not shown).

The top gene in Table 2 showing positive regulation by FinP RNA (*cysD*) was chosen for further study. Aside from its top position in the list, an additional reason for the choice was that *ygaE* and *STM4302* are uncharacterized *Salmonella* loci while *cysD* is a well known biosynthetic gene (Tei *et al.*, 1990). CysD is the catalytic subunit of sulfate adenylyltransferase (Leyh *et al.*, 1992), an enzyme of the so-called "sulfate reduction pathway" whose final product is sulfide (Kredich, 1996).

Gene	ID	Fold regulation[a]	Description[b]
cysD	STM2935	+44.85	ATP sulfurylase, subunit 1
ygaE	STM2794	+ 4.01	putative transcriptional repressor
dadX	STM1802	+ 3.75	alanine racemase 2
oppB	STM1745	+ 3.61	oligopeptide transport protein
STM4302	STM4302	- 9.44	putative cytoplasmic protein

Table 2. Chromosomal genes differentially regulated upon pKP32-3 expression
[a] Fold-regulation obtained by transcriptome analysis following induction of *finP* on *Salmonella* whole genome microarrays ("SALSA"). Listed are genes that were at least 3-fold differentially regulated and statistically significant using SAM (Tusher et al., 2001)
[b] Description based on the annotation found at Colibase (http://xbase.bham.ac.uk/colibase/)

Correlation between FinP production, *cysD* mRNA abundance and CysD protein level. Levels of *cysD* mRNA were monitored in the presence of different levels of FinP RNA, expressed from the arabinose-dependent promoter of plasmid pKP32-3. For this purpose, strain SV5505 was grown in LB supplemented with arabinose (either 0.2%, 0.4%, or 0.5%) and in LB as a control. Using quantitative RT-PCR, FinP RNA levels and *cysD* mRNA levels were tested in aliquots from both cultures. A direct correlation between the amounts of FinP RNA and *cysD* mRNA was found (Fig. 2). This observation provided further support that FinP RNA increased *cysD* mRNA content.

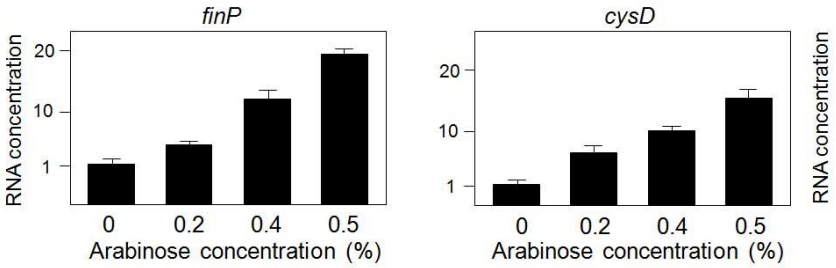

Figure 2. Relative amounts of FinP RNA and *cysD* mRNA of SV5505 strain, normalized to *rfaH* mRNA. The presence or absence of arabinose in the culture medium permitted regulated synthesis of FinP RNA, expressed from the arabinose-dependent promoter of pKP32-3. Each bar represents the average from three independent experiments.

Western blotting was used to determine whether FinP affected the level of CysD protein. For this purpose, strain SV5722 was grown in LB until early stationary phase ($OD_{600} \approx 1$). Arabinose was then added to obtain a final concentration of

0.2%. After 30 min, cells were harvested, and Western hybridization using a commercial anti-FLAG antibody was performed. These experiments indicated that a relatively low concentration of arabinose (0.2%) was sufficient to observe an increase in CysD protein (Fig. 3), providing further evidence that FinP RNA exerts positive control of *cysD* gene expression.

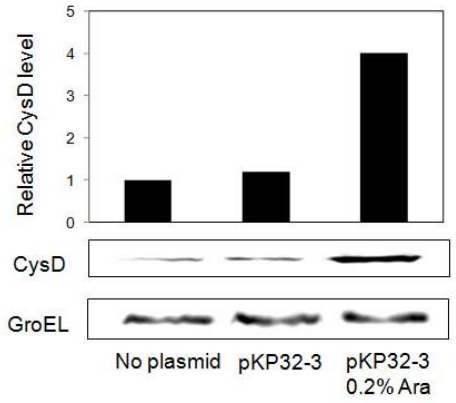

Figure 3. Levels of CysD protein in protein extracts from strain SV5722 (*cysD*::3xFLAG / pKP32-3) grown with or without L-arabinose. An extract from SV5507 (*cysD*::3xFLAG) was also included as control. Western blotting was performed with anti-FLAG and anti-GroEL commercial antibodies. Quantitation of CysD levels was obtained with Image Gauge software.

FinP RNA regulates the entire *cysDNC* operon. Because the *cysD* gene is part of an operon that includes also *cysN* and *cysC* (Kredich, 1996), we examined whether the downstream *cysN* and *cysC* genes were also under FinP RNA control. Two kinds of experiments were performed:

(i) Levels of *cysN and cysC* mRNAs were monitored by quantitative RT-PCR in the presence of FinP RNA, expressed from the arabinose-dependent promoter of plasmid pKP32-3 as above. The *cysC* gene is known to be expressed at a lower level than *cysD* and *cysN* (Leyh *et al.*, 1992), presumably because of natural polarity (Li & Altman, 2004). Hence, we increased the sensitivity of the assay by using a relatively high arabinose concentration (0.4%). The relative levels of *cysD*, *cysN*, and *cysC* mRNAs under such conditions are shown in Fig. 4. A polarity gradient is observed indeed. However, the most relevant conclusion from this experiment was that FinP RNA increases the concentration of all three ORFs in the *cysDNC* transcript. The unequivocal conclusion from these experiments is that FinP RNA may have a positive effect on the entire *cysDNC* transcript.

(ii) Western blotting was used to determine whether FinP affected the level of CysN protein. A Western blot analysis of CysD and CysN levels upon induction of finP expression with 0.4% arabinose is shown in Fig. 4. Unfortunately, the CysC protein could not be visualized by Western blotting. Increased amounts of CysD and CysN proteins were detected upon FinP overproduction. This finding may be especially

relevant, since CysD and CysN are subunits of the same enzyme, sulfate adenylyltransferase (Kredich, 1996; Leyh *et al.*, 1992).

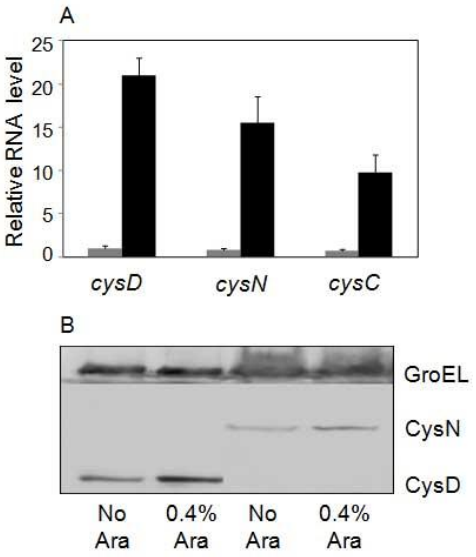

Figure 4. A. Relative amounts of *cysD, cysN, and cysC* mRNAs, normalized to *rfaH* mRNA, in the absence of arabinose (grey histograms) and in the presence of 0.4 % arabinose (black histograms). FinP RNA was expressed from pKP32-3. Data are averages from three independent experiments. **B**. Levels of CysD and CysN proteins in protein extracts from strains SV5722 (*cysD*::3xFLAG / pKP32-3) and SV5723 (*cysN*::3xFLAG /pKP32-3) grown with or without arabinose. Western blotting was performed with anti-FLAG and anti-GroEL commercial antibodies. Quantitation of CysD levels was obtained with Image Gauge software.

Effect of *cysD* disruption on conjugal transfer of pSLT. The evidence that certain chromosomal genes are under the control of FinP RNA raised the intriguing possibility that these genes might play a role in conjugation. A null *cysD* allele was constructed, and the frequencies of pSLT conjugal transfer were compared using isogenic donors: SV5556 (*spvA*::Tn*5d*Km), SV5729 (*spvA*::Tn*5d*Km Δ*cysD*). The *spvA*::Tn*5d*Km allele provided a pSLT-borne marker for the selection of transconjugants, as previously described (Garcia-Quintanilla *et al.*, 2008). The recipient was SV5534 in all cases. Matings were carried in LB agar at 37ºC in anaerobic GasPak jars, and were allowed to proceed for 4 h. Mating mixtures were then diluted, and spread on LB supplemented with kanamycin (for pSLT selection) and chloramphenicol (for donor counterselection). To our surprise, knockout of *cysD* increased the conjugation frequency above one order of magnitude (Fig. 5). Usually, deletions generated by the Datsenko and Wanner procedure are partially polar (Datsenko & Wanner, 2000); hence, decreased expression of downstream genes *cysN* and *cysC* could contribute to reduction of conjugal transfer (see below). Aside from this caveat, these experiments suggested that CysD is an inhibitor of conjugation (alone or together with the CysD and CysN gene products). No evidence indicating an involvement, direct or indirect, of CysD or other functions of the sulfate reduction pathway in control of conjugation had been previously reported.

The above experiment raised the question of whether inhibition of conjugation was a specific trait of the *cysDNC* operon or a general property of genes involved in the

sulfate reduction pathway. To address this question, a *cysI asrC* double mutant was constructed (strain SV5795). CysI is a subunit of NADPH-sulfite reductase, a multimeric enzyme that catalyzes conversion of sulfite to sulfide (Kredich, 1996). AsrC is a CysI homolog that performs the same reaction under anaerobic conditions (Huang & Barrett, 1991). Because pSLT transfer occurs at higher rates under microaerophilia (Garcia-Quintanilla *et al.*, 2008), knock-out of both homologs was judged advisable. Matings were performed as above except that SV5795 (*ΔcysI ΔasrC*) was used as donor. A 10 fold increase in the frequency of conjugal transfer was observed (Fig. 5), suggesting that an active sulfate reduction pathway reduces conjugal transfer indeed.

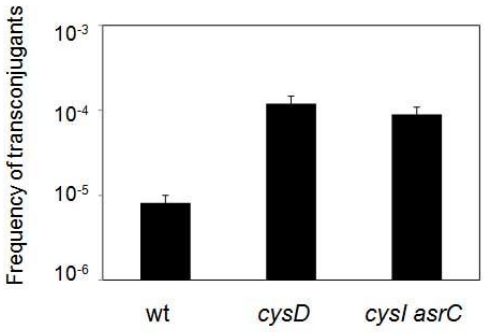

Figure 5. Effect of *cysD* and *cys asrC* mutations on conjugal transfer of pSLT. Donors were SV5556, SV5729 (*ΔcysD*), and SV5795 (Δ*cysI ΔasrC*). The recipient was SV5534 in all cases. Data are averages and standard deviations from 8 independent matings.

Effect of sulfur metabolism on conjugal transfer. Increase of conjugal transfer by mutations in the sulfate reduction pathway raised the unsuspected possibility that metabolites of the L-cysteine biosynthetic pathway could serve as signals for the control of plasmid transfer. To test this hypothesis, matings were carried out in the presence of sodium sulfide, the final product of the sultate reduction pathway (Kredich, 1996), and L-cysteine, the final product of the cysteine biosynthetic pathway. Matings were performed as above, except that the mating plates contained either sodium sulfide or cysteine. As controls, matings were also carried out in the presence of a sulfur-containing amino acid (L-methionine) and an additional, sulfurless amino acid (L-threonine). Amino acid supplements were added at the final concentration of 15 mM. Sodium sulfide was added at the final concentration of 30 mM. The results (Fig, 6, panel A) can be summarized as follows: (i) The presence of cysteine caused a spectacular decrease in the frequency of conjugation (near two orders of magnitude); (ii) a smaller but significant decrease (around 10 fold) was observed in the presence of sodium sulfide; (iii) neither L-methionine nor L-threonine affected the conjugation frequency, indicating the inhibitory effects of L-cysteine and of its precursor sulfide are specific.

Inhibition of conjugal transfer by L-cysteine was also observed at lower concentrations, and the magnitude of the effect was roughly proportional to the concentration of the inhibitor (Fig. 6, panel B). To a lesser extent, a similar effect was observed with sodium sulfide (data not shown). Reduction of conjugal transfer in the presence cysteine was likewise observed when a *cysD* mutant was used as donor (Fig. 6, panel C), suggesting that L-cysteine can exert its inhibitory effect even in the absence of active biosynthesis. Altogether, these observations suggest a dose-dependent response of the conjugation machinery to the presence L-cysteine, which acts as an inhibitor of mating.

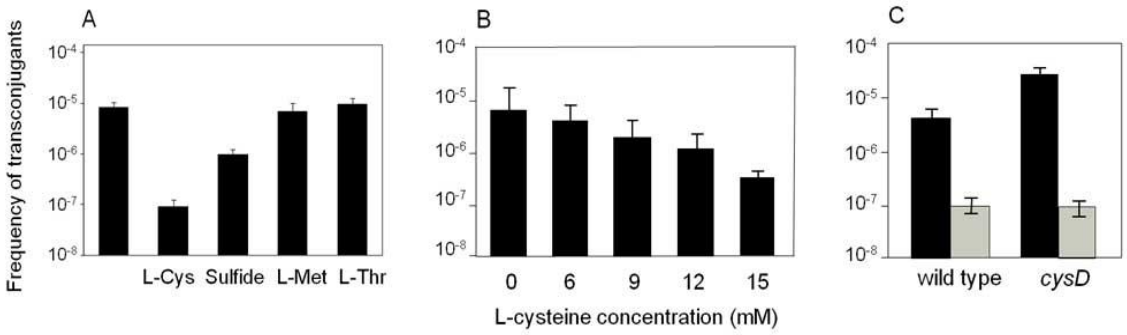

Figure 6. **A**. Effect of L-cysteine, sodium sulfide, L-methionine, and L-threonine on conjugal transfer of pSLT, using SV5556 as donor, and SV5534 as recipient. Data are averages and standard deviations from 8 independent matings. **B.** Effect of various L-cysteine concentrations on conjugal transfer of pSLT, using SV5556 as donor, and SV5534 as recipient. Data are averages and standard deviations from >3 independent matings. **C**. Effect of L-cysteine in conjugal transfer of pSLT from *cys*$^+$ and *cys* donors (SV5556 and SV5729, respectively). The recipient was SV5534 in all cases. Black histograms represent frequencies of mating in LB, while grey histograms represent frequencies of mating in LB supplemented with 15 mM L-cysteine. Data are averages and standard deviations from 8 independent matings.

DISCUSSION

The existence of bacteriophage-encoded products that modulate the expression of host genes has been known for decades (Boeckh *et al.*, 1986; Bunny *et al.*, 2002; Duckworth, 1970; Nechaev & Severinov, 2003). In contrast, the literature contains few examples of plasmid functions able to modulate host gene expression. Exceptions are the addiction modules that target host functions upon plasmid loss (Zielenkiewicz & Ceglowski, 2001) and a plasmid-encoded RNA that controls tryptophanase activity in *E. coli* (Chant & Summers, 2007). In this study we provide evidence that FinP RNA, a small RNA encoded by F-like plasmids

including the *Salmonella* virulence plasmid (Finlay *et al.*, 1986a; Finlay *et al.*, 1986b; Rotger & Casadesus, 1999), regulates chromosomal loci, either increasing their expression (*ygaE* and *cysD*) or decreasing it (*STM4302*) (Table 2). Although the underlying mechanisms remain to be investigated, a possibility is that FinP RNA might interact with additional mRNA molecules besides *traJ* mRNA. The abundance of FinP RNA in the cell (Figure 1) might facilitate these "secondary" interactions, perhaps affecting mRNA stability and/or mRNA function as described for many small regulatory RNAs [reviewed in (Waters & Storz, 2009)]. However, interactions between bacterial small RNAs and proteins have been also described (Lapouge *et al.*, 2008; Lenz *et al.*, 2005; Romeo, 1998; Wassarman & Storz, 2000).

The observation that FinP RNA increased the expression of the biosynthetic operon *cysDNC* (Figures 2, 3, and 4) raised the possibility, at first sight odd, that enzymes of the L-cysteine biosynthetic pathway might play a role in mating. Genetic evidence confirmed that L-cysteine biosynthesis affects conjugal transfer: *cysD* and *cysI asrC* strains of *S. enterica,* which lack enzymes of the sulfate reduction pathway, undergo conjugation at increased rates (Figure 5). Two additional observations argue in favor of the involvement of L-cysteine in conjugation control. First, exogenous sulfide (the final product of the sulfate reduction pathway) and L-cysteine reduce conjugal transfer of pSLT (Fig. 6, panels A and B) if provided at the standard concentrations used in physiological studies (Awano *et al.*, 2005; Yamada *et al.*, 2006). Second, sulfide and L-cysteine inhibit conjugal transfer not only in the wild type but also in a *cysD* donor (Fig. 6, panel C), suggesting that inhibition does involve L-cysteine biosynthesis, and not a secondary activity of the *cysDNC* gene products.

We do not understand why L-cysteine is an inhibitor of conjugal transfer. However, previous studies have shown that L-cysteine can serve as a signal for specific bacterial processes. In *S.* Typhimurium, mutations in *cysB* and *cysE* confer resistance to mecillinam, and thiosulfate, sulfite, sulfide and cysteine abolish this resistance (Oppezzo & Anton, 1995). L-cysteine also regulates swarming motility (Burkart *et al.*, 1998; Kim & Surette, 2003; Toguchi *et al.*, 2000). In *Bordetella pertussis,* the availability of cysteine regulates toxin expression (Bogdan *et al.*, 2001), and genes of the sulfur assimilatory pathway are required for *Brucella melitensis* virulence (Lestrate *et al.*, 2000). In *Mycobacterium tuberculosis,* sulfur metabolism has been implicated in virulence, antibiotic resistance and anti-oxidant defense, and the *cysDNC* genes are induced upon exposure to oxidative stress

(Pinto *et al.*, 2004). Biofilm formation by *E. coli* occurs at an accelerated rate in *cysB* (Ren *et al.*, 2005) and *cysE* mutants (Sturgill *et al.*, 2004). In a *cysE* mutant, addition of cysteine reduces biofilm formation 2.5 fold, suggesting that cysteine might be a signal that inhibits biofilm development (Sturgill *et al.*, 2004).

The above examples from the literature raise the possibility that certain sulfur-containing compounds such as L-cysteine and sulfide might likewise serve as mating inhibition signals in certain environments. One such environment might be the large intestine, where conjugal transfer of pSLT is known to be inhibited (Garcia-Quintanilla *et al.*, 2008). Sulfide is known to be synthesized by the microbiota of the large intestine, using oxidized sulfur compounds present in food as well as endogenous sulfated mucins (Shoveller *et al.*, 2005). Under such conditions, FinP-mediated increase of *cysDNC* expression might compensate for feedback inhibition of the sulfate reduction pathway (Kredich, 1996), thus contributing to sustain high levels of L-cysteine biosynthesis. An alternative possibility is that the sulfate reduction pathway of *S. enterica* might be activated in response to toxic oxidants as described in *Mycobacterium tuberculosis* (Pinto *et al.*, 2004). Oxidative damage caused by bile salts (Prieto *et al.*, 2006) might activate the *Salmonella* sulfate reduction pathway in the duodenum, thus producing inhibitory signals for conjugation. Transfer of pSLT transfer is inhibited in the duodenum (Garcia-Quintanilla *et al.*, 2008), and FinP-mediated enhancement of *cysDNC* expression might contribute to conjugal inhibition. Finally, *S. enterica* requires increased L-cysteine synthesis for swarming motility (Turnbull & Surette, 2008). Swarming and biofilm formation are mutually exclusive in *S. enterica* (Harshey, 2003; Mireles *et al.*, 2001), and evidence exists that biofilm formation may favor mating in natural environments (Ghigo, 2001; Hausner & Wuertz, 1999; Molin & Tolker-Nielsen, 2003). Hence, a conceivable scenario is that high concentrations of L-cysteine might favor swarming motility, which in turn would prevent conjugal transfer.

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Chapter 4: Virulence plasmid interchange between strains ATCC 14028, LT2 and SL1344 of *Salmonella enterica* serovar Typhimurium

Virulence plasmid interchange between strains ATCC 14028, LT2 and SL1344 of *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Strains ATCC 14028 and SL1344 of *Salmonella enterica* serovar Typhimurium are more virulent than LT2 in the BALB/c mouse model. Virulence plasmid swapping between strains ATCC 14208, LT2, and SL1344 does not alter their competitive indexes during mouse infection, indicating that the three plasmids are functionally equivalent, and that their contribution to virulence is independent from the host background. Strains ATCC 14028 and LT2 are more efficient than SL1344 as conjugal donors of the virulence plasmid. Virulence plasmid swapping indicates that reduced ability of conjugal transfer is a property of the SL1344 plasmid, not of the host strain. An A → V amino acid substitution in the TraG protein appears to be the major cause that reduces conjugal transfer in the virulence plasmid of SL1344. Additional sequence differences in the *tra* operon are found between the SL1344 plasmid and the ATCC 14028 and LT2 plasmids. Divergence in the *tra* operon may reflect the occurrence of genetic drift either after laboratory domestication or in the environment. The latter might provide evidence that possession of conjugal transfer functions is a neutral trait in *Salmonella* populations, a view consistent with the abundance of *Salmonella* isolates whose virulence plasmids are non-conjugative.

INTRODUCTION

LT2, ATCC 14028 and SL1344 are three model strains of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). Since its isolation in the 1940s by Lilleengen, *S.* Typhimurium LT2 has been widely used in genetic studies (Lilleengen, 1948; Sanderson, 1987; Zinder, 1952). The descendants of the original LT2 isolate are most accurately described as a set of clades, most of which are highly attenuated for virulence in the mouse model due to an *rpoS* mutation (Bearson *et al.*, 1996; Sanderson, 1987; Swords *et al.*, 1997). This fact has led many investigators to use other *S.* Typhimurium virulent strains to study *Salmonella* pathogenesis (Fields *et al.*, 1986; Gulig & Curtiss, 1987; Harrington & Hormaeche, 1986). SL1344, isolated by B.A.D. Stocker, and ATCC 14028, descendant of a strain isolated from chickens in 1960 (CDC 60-6516), are two popular mouse-virulent strains.

The main chromosomal differences between the three model strains involve the presence or absence of prophages. The three strains are lysogenic for Gifsy-1 and Gifsy-2 (Figueroa-Bossi *et al.*, 2001). ATCC 14028 and SL1344 also carry the cryptic St64B prophage (Figueroa-Bossi & Bossi, 2004). Furthermore, each strain carries specific prophages. Fels-1 and Fels-2 are present only in LT2, although there are remnants of Fels-2 genes in the ATCC 14028 genome (Figueroa-Bossi *et al.*, 2001). Prohage Gifsy-3 is only present in strain ATCC 14028 while SL1344 carries SopEΦ (Figueroa-Bossi *et al.*, 2001). SL1344 displays additional strain-specific phenotypes such as histidine auxotrophy, streptomycin resistance, and inability to form biofilms (Garcia *et al.*, 2004; Hoiseth & Stocker, 1981).

Certain serovars of *Salmonella enterica* belonging to subspecies I, particularly those showing host adaptation (Paratyphi C, Enteritidis, Dublin, Choleraesuis, Gallinarum/Pullorum, Abortusovis and Typhimurium) carry a plasmid of 50-100 kb known as the "*Salmonella* virulence plasmid" (Baumler *et al.*, 1998; Guiney *et al.*, 1994; Gulig *et al.*, 1993). All *Salmonella* virulence plasmids share a 7.8-kb region, *spv* (*Salmonella* plasmid virulence), required for bacterial proliferation in the mononuclear phagocytic system of warm-blooded vertebrates (Gulig *et al.*, 1993; Rotger & Casadesus, 1999). Futhermore, hybrid virulence-resistance (VR) plasmids containing both virulence and antibiotic resistance determinants have been detected in clinical isolates of *Salmonella enterica* (Guerra *et al.*, 2002; Mendoza Mdel *et al.*, 2009).

The presence of transfer (*tra*) genes in the virulence plasmids of all serovars suggests that a *Salmonella* ancestor acquired the virulence plasmid by conjugation, and that divergence has occurred during the evolution of the serovars (Rotger & Casadesus, 1999). The serovar-specific plasmid of *S.* Typhimurium contains a complete *tra* operon (see below). In other serovars, the virulence plasmid has suffered deletions. However, some of these plasmids still contain the origin of transfer, *oriT*, and are mobilizable (Barrow & Lovell, 1989; Chu *et al.*, 2002; Ou *et al.*, 1994).

The virulence plasmid of *S. enterica* serovar Typhimurium strain LT2 was designated pSLT in 1982 (Jones *et al.*, 1982). However, this 93 kb plasmid had already been described in 1970 (Dowman & Meynell, 1970). Plasmid pSLT carries a complete *tra* operon similar to that of the F sex factor and belongs to the MOB_{F1} group on the basis of relaxase classification (Garcillan-Barcia et al., 2009). Other well-known F-like plasmids such as R1, R100 (NR1) and F plasmid are also members of this family. The ability of pSLT to reduce conjugal transfer of the F sex factor was described in the 1970's (Smith et al., 1973, Spratt, 1973 #496). Further studies showed that a wild type FinOP system of fertility inhibition present in pSLT was able to complement in *trans* the *finO* mutation of F, thus reducing expression of the F transfer operon (Sanderson et al., 1983). However, pSLT was thought to be nonconjugative until 1999, when B. Ahmer and F. Heffron demonstrated that the virulence plasmids of ATCC 14028 and LT2 were self-transmissible (Ahmer *et al.*, 1999). However, these authors were not able to detect conjugation when the SL1344 strain was used as donor (Ahmer *et al.*, 1999).

In this study, we show that the virulence plasmids of strains LT2, ATCC 14028 and SL1344 are all self-transmissible albeit at different frequencies. We also show that the conjugal donor capacity of each strain is a plasmid-borne trait, regardless of the host background. An A → V amino acid substitution in the TraG protein appears to be the major factor that reduces conjugal transfer in the virulence plasmid of SL1344, thus explaining the difficulty to detect conjugal transfer in this strain (Ahmer et al., 1999). Additional nucleotide sequence differences that do not impair conjugal transfer are found between the virulence plasmids of strains LT2 and SL1344. In contrast, the virulence plasmids are interchangeable for virulence, a trait that correlates with the absence of polymorphism in their *spv* regions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The strains of *Salmonella enterica* used in this study (Table 1) belong to serovar Typhimurium. Some strains derive from the mouse-virulent strains ATCC 14028 and SL1344, and others from the classical strain LT2. *E. coli* CC118 λ *pir* (Herrero *et al.*, 1990) and *E. coli* S17-1 λ *pir* (Simon, 1983) were kindly provided by Victor de Lorenzo, CNB, CSIC, Cantoblanco, Spain. Transductional crosses using phage P22 HT 105/1 *int201* (Schmieger, 1972) were used for strain construction operations involving chromosomal markers. The transduction protocol has been described elsewhere (Garzon *et al.*, 1995). To obtain phage-free isolates, transductants were purified by streaking on green plates (Chan *et al.*, 1972). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. The *trg*::Mu*d*Q allele is neutral for *Salmonella* conjugation and provides a marker for strain discrimination in mating (Garcia-Quintanilla *et al.*, 2008). Strains SV4937, SV4038 and SV4039 were constructed by transducing the *trg*::Mu*d*Q (Cm^r) allele from SV4984, an ATCC 14028 derivative.

Strains	Genotype
ATCC 14028[1]	Wild type
LT2[2]	Wild type
SL1344[3]	Wild type *hisG*
SV4478[4]	LT2 *ΔfinO*::Km
SV4934	14028 *ΔfinO*::Km
SV4935	SL1344 *ΔfinO*::Km
SV4937	14028 *trg*:: mu*d*Q (Cm^r) $pSLT^-$
SV4938	SL1344 *trg*:: mu*d*Q (Cm^r) $pSLT^-$
SV4939	LT2 *trg*:: mu*d*Q (Cm^r) $pSLT^-$
SV5021	LT2 / $pSLT^{14028}$ *ΔfinO*::Km
SV5022	LT2 / $pSLT^{1344}$ *ΔfinO*::Km
SV5069	SL1344 / $pSLT^{14028}$ *ΔfinO*::Km
SV5073	SL1344 / $pSLT^{LT2}$ *ΔfinO*::Km
SV5499	14028 / $pSLT^{LT2}$ *ΔfinO*::Km
SV5501	14028 / $pSLT^{1344}$ *ΔfinO*::Km
SV5522	LT2 / *ΔfinO*::Km *traA*-SL1344[a]
SV5523	LT2 / *ΔfinO*::Km *srgB*-SL1344[b]

SV5524	LT2 / *ΔfinO*::Km *traG*-SL1344[c]
SV5525	SL1344 / *ΔfinO*::Km *traA*-LT2[d]
SV5526	SL1344 / *ΔfinO*::Km *srgB*-LT2[e]
SV5527	SL1344 / *ΔfinO*::Km *traG*-LT2[f]
SV5528	14028 / *ΔfinO*::Km *traG*-SL1344[c]

Table 1. Strain list
1. (Fields *et al.*, 1986)
2. Salmonella Genetic Stock Centre, University of Calgary, Alberta, Canada
3. (Hoiseth & Stocker, 1981)
4. (Camacho & Casadesus, 2005)
a. *traA*-SL1344 designates the SL1344 *traA* allele. Unlike the LT2 *traA* allele which carries Phe at position 5, *traA*-SL1344 contains Leu. The amino acid change is due to a single nucleotide change that changes a TTA codon to TTT.
b. *srgB* H90P: CAC → CCC
c. *traG* A302V: GCC → GTC
d. *traA* L5F: TTT → TTA
e. *srgB* P90H: CCC → CAC
f. *traG* V302A: GTC → GCC

Media and chemicals. E medium (Vogel & Bonner, 1956) was used as minimal medium for *S. enterica*. The rich medium was Luria-Bertani (LB). Nutrient broth was from Difco (Voight Global Distribution, Lawrence, Kansas), supplemented with 5% sucrose (Panreac, Castellar del Vallès, Spain). Solid media contained agar at 1.5% final concentration. Green plates were prepared according to the original recipe (Chan *et al.*, 1972), except that methyl blue (Sigma-Aldrich, St. Louis, Missouri) substituted for aniline blue. Antibiotics were used at the final concentrations described by Maloy (Maloy, 1990).

Construction of S. enterica strains carrying heterologous virulence plasmids. Derivatives of ATCC 14028, LT2, and SL1344 cured from their virulence plasmids (strains SV4937, SV4938, and SV4939) were used as recipients in conjugation crosses. Donors were proline auxotrophs carrying *ΔfinO*::Km^r virulence plasmid derivatives. Transconjugants were selected on E plates supplemented with kanamycin.

Construction of FinO$^-$ derivatives of the ATCC14028 and SL1344 virulence plasmids. The *finO* locus of the virulence plasmids of ATCC14028 and SL1344 was knocked out by adapting to *S. enterica* the method of Datsenko and Wanner

(Datsenko & Wanner, 2000). Primers for PCR amplification and deletion verification were as previously described (Camacho & Casadesus, 2002; Garcia-Quintanilla *et al.*, 2006).

Allele interchanges. The *traA*, *srgB* and *traG* genes from strain LT2 and SL1344 of *S. enterica* serovar Typhimurium were PCR-amplified using two 30-nucleotide primers that contained KpnI and SacI targets, respectively. The primers for *traA* amplification were 5´ TCA TGG TAC CTC ATT CTG GAA CGA ACT GTC 3´ and 5´ TCG TGA GCT CCC AGA CTG AGC ACA ATC AGC 3´; for *srgB*, 5´ TCA AGG TAC CCT GAC TAT CCA CAG CGT GTC 3´ and 5´ TAC TGA GCT CCA TGG TGG CAT TAC AGG AAC 3´; and for *traG*, 5´ TAC TGG TAC CGA CCA TAC GGA CAT CAG TAC 3´ and 5´ TAG TGA GCT CTA CCG GTC TGG TAA GTC ATC 3´. The resulting fragments were digested with KpnI and SacI, cloned onto pDMS197 (Edwards *et al.*, 1998), and propagated in *E. coli* CC118 λ *pir* (Herrero et al., 1990). Clones derived from pMDS197 were transformed into *E. coli* S17-1 λ *pir* (Simon, 1983). The resulting strains were used as donors in matings with *S. enterica* LT2 or SL1344 as receptors, depending on the case, selecting Tc^r transconjugants on E medium plates supplemented with tetracycline (5 mg/l). Several Tc^r transconjugants were grown in nutrient broth (without NaCl) containing 5% sucrose. Individual tetracycline-sensitive segregants were then examined for the incorporation of the heterologous allele. Replacements of *traA*, *srgB* and *traG* were confirmed by DNA sequencing.

Competitive index (CI) virulence assays. Eight-week-old female BALB/c mice were used for virulence tests. Groups of three animals were inoculated with a 1:1 ratio of two strains. Bacterial cultures were grown overnight at 37°C in LB without shaking. Feeding of mice was suspended 1 day before inoculation. Oral inoculation was performed by feeding the mice with 25 µl of saline (0.9% NaCl) containing 0.1% lactose and $5 \cdot 10^7$ bacterial CFU of each strain. Bacteria were recovered from the mouse spleens 6 days after inoculation, and the CFU were enumerated on appropriate media. A competitive index (CI) for each mutant was calculated as the ratio between the wild type and the mutant strain in the output divided by their ratio in the input (Beuzon & Holden, 2001). Student's *t* test was used to analyze CIs. *P* values of 0.05 or less were considered significant.

Bacterial matings. Cultures of the donor and the recipient were prepared in minimal medium. Cells were harvested by centrifugation and washed with 10mM $MgSO_4$. Aliquots of both strains, 500 µl each, were then mixed and centrifuged for 30 s at 15000 g. The pellet was resuspended in 20 µl of 10mM $MgSO_4$ and sucked onto a Millipore filter, 0.45 µm pore size. The filters were placed on E plates without glucose and incubated at 37°C for 4 h. After mating, the mixtures were diluted in 10mM $MgSO_4$ and spread on selective plates. As controls, 0.1 ml of both the donor and the recipient cultures were also spread on selective plates. Conjugation frequencies were calculated per donor bacterium.

Nucleotide sequence analysis. The SL1344 sequence was obtained from the Sanger Institute (Hinxton, UK) website (http://www.sanger.ac.uk/Projects/Salmonella/). Using the BLAST service provided at the Sanger Institute, the available nucleotide sequence of $pSLT^{1344}$ was compared with the complete $pSLT^{LT2}$ nucleotide sequence which is available at GenBank under accession number AE006471.

RESULTS

pSLT is self-transmissible in strain SL1344. Even though a previous study had been unable to detect conjugal transfer of the virulence plasmid of SL1344 (Ahmer et al., 1999), we considered the possibility that $pSLT^{1344}$ might be conjugative at very low frequencies. Hence, we assayed $pSLT^{1344}$ transfer under optimal conditions for the detection of transconjugants. Because lack of FinO had been previously shown to increase between 10^2 and 10^3 fold the frequency of pSLT transfer (Camacho & Casadesus, 2002; Garcia-Quintanilla *et al.*, 2008) we used a *ΔfinO* derivative of $pSLT^{1344}$. Furthermore, matings were performed on minimal E medium, previously shown to permit higher rates of conjugal transfer than rich medium (Camacho and Casadesus, 2002). The results showed that a *ΔfinO* of derivative $pSLT^{1344}$ is self-transmissible, albeit at frequencies nearly two orders of magnitude lower than $pSLT^{LT2}$ *ΔfinO* and three orders of magnitude lower than $pSLT^{14028}$ *ΔfinO* (Fig. 1).

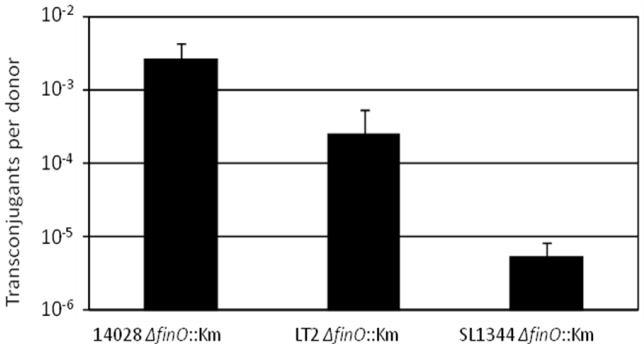
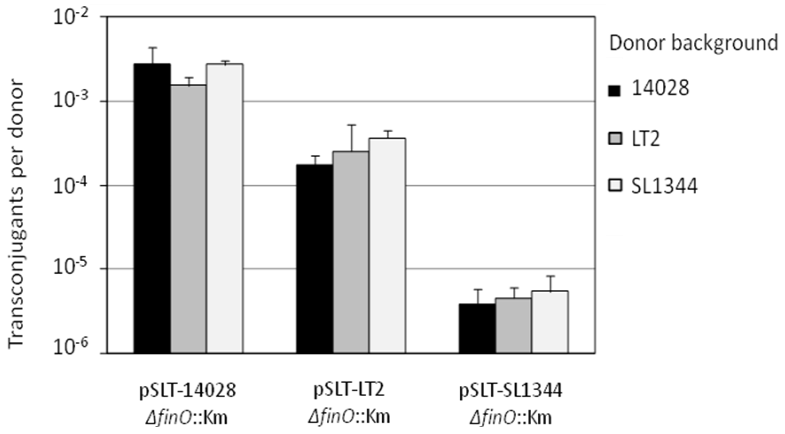


Figure 1. Conjugal transfer of pSLT *ΔfinO* derivatives. Donors are indicated below the histograms. Crosses were as follows: donor SV4934 (14028 *ΔfinO*::Km) and recipient SV4937; donor SV4478 (LT2 *ΔfinO*::Km) and recipient SV4939; donor SV4935 (SL1344 *ΔfinO*::Km) and recipient SV4938. Frequencies were calculated as number of transconjugants per donor. Values are averages and standard deviations from 12 independent matings.

Differences in conjugal transfer frequency are a property of each pSLT plasmid, not of the host strain. Because the three strains under study differed in their ability to act as conjugal donors of the virulence plasmid, we examined whether the differences were plasmid-borne or caused by the host background. Note that conjugal transfer of plasmids is under the control of a large number of chromosomal regulators (Frost & Koraimann, 2010), and differences in such regulators might potentially affect pSLT gene expression. We constructed strains carrying heterologous pSLT plasmids and used them as donors in mating experiments. The recipients were cured derivatives of ATCC 14028, LT2, and SL1344 (SV4937, SV4939, and SV4938, respectively). The frequency of conjugation was found to depend on the plasmid only, not on the host background (Fig. 2, panel A). Moreover, the frequencies of mating were also independent from the recipient strain (Fig. 2, panel B).

A



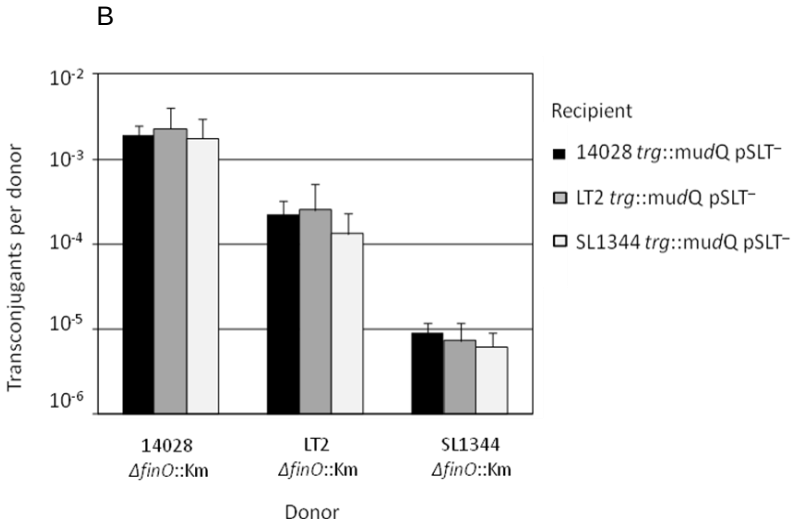


Figure 2. A. Frequencies of conjugal transfer of pSLT *ΔfinO* derivatives in different donor backgrounds. Histogram colors identify the chromosomal background: black, ATCC 14028; grey, LT2; white, SL1344. In all crosses involving pSLT^{14028}, the recipient was SV4937. In turn, the recipient of pSLT^{LT2} was SV4939, and the recipient of pSLT^{1344} was SV4938. **B.** Effect of the recipient strain on conjugal transfer of pSLT. Histogram colors indicate the recipient strain: black, ATCC 14028 *trg*::mu*d*Q pSLT$^-$ (SV4937); grey, LT2 *trg*::mu*d*Q pSLT$^-$ (SV4939); white, SL1344 *trg*::mu*d*Q pSLT$^-$ (SV4938). In both panels, frequencies are averages and standard deviations from 8 independent matings.

A single mutation in the *traG* gene contributes to the low transfer frequency of pSLT^{1344}. Nucleotide sequence comparisons between pSLT^{LT2} and pSLT^{1344} showed differences in a number of sites, including three genes involved in conjugal transfer: *traG*, *traA* and *srgB* (Table 2). TraG is involved in pilus tip formation (Firth & Skurray, 1992), exclusion of DNA entry into the recipient cell (Audette et al., 2007), and mating pair stabilization (Firth & Skurray, 1992). The *traA* gene encodes propilin subunits (Majdalani & Ippen-Ihler, 1996; Majdalani *et al.*, 1996). The function of *srgB* (*sdiA*-regulated gene) is unknown. However, the fact that *srg* transcription is under the control of SdiA, a LuxR family member, has raised the possibility that the SrgB protein might be involved in mating.

Allele interchanges were performed between the *traG, traA*, and *srgB* genes of pSLT^{LT2} and pSLT^{1344}. The resulting strains were then used as donors in matings with a pSLT-cured strain. Mating design and calculation of conjugation frequencies were as above. The results were unambiguous: the C → T nucleotide substitution found in the *traG* gene of pSLT^{1344} caused a strong reduction in conjugal transfer when introduced in pSLT^{LT2} (Figure 3). In turn, when the *traG* sequence of pSLT^{LT2} was re-constructed in SL1344, transfer of pSLT^{1344} increased (Figure 3). These

observations indicated that the A → V amino acid substitution predicted to occur in the TraG protein of pSLT[1344] decreases its ability to promote conjugal transfer. In contrast, the allelic exchanges performed in *traA* and *srgB* did not alter transfer frequencies in the resulting plasmids (Figure 3).

Gene	Size (aa*)	Change (aa)	LT2	SL1344	Function
srgB	299	90	CAC (His)	CCC (Pro)	Unknown
traA	120	5	TTA (Phe)	TTT (Leu)	Propilin
traG	940	302	GCC (Ala)	GTC (Val)	N_t: pilus tip formation aa 610-673: DNA entry exclusion Entire protein: mating pair stabilization

Table 2. Mating-related genes whose sequences are different in the virulence plasmids of LT2 and SL1344
* aa: amino acid

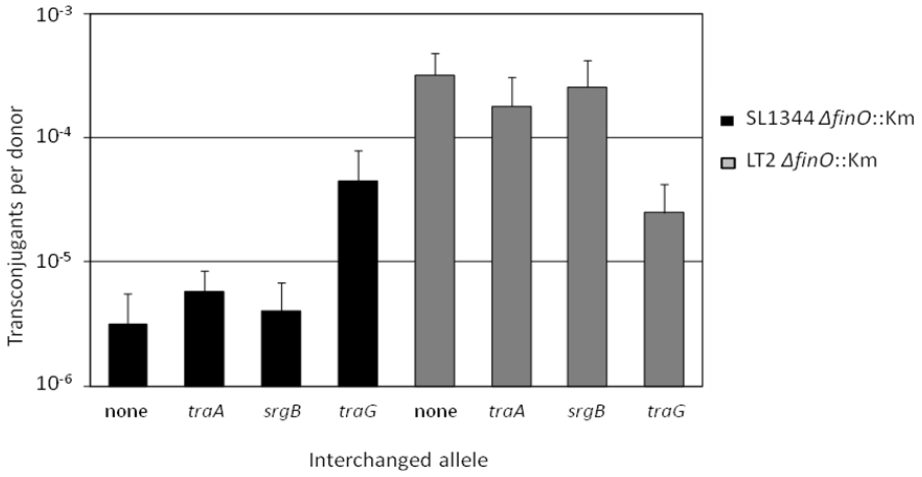


Figure 3. Effect of *traA*, *srgB* and *traG* allele interchanges on conjugation frequencies. Histogram colors indicate the donor: black, SL1344 *ΔfinO*::Km; grey, LT2 *ΔfinO*::Km. The relevant genotype of each donor is indicated below the corresponding histogram. The recipient was SV4939 in all matings. Frequencies are averages and standard deviations from 8 independent matings.

Interchange of pSLT does not alter Salmonella virulence. The observation that $pSLT^{LT2}$, $pSLT^{1344}$ and $pSLT^{14028}$ differ in their ability to promote conjugation, combined with the fact that LT2 is attenuated in mice due to a mutation in *rpoS* (Bearson et al., 1996; Sanderson, 1987; Swords et al., 1997), raised the question of whether the virulence plasmids might also differ in their contribution to virulence. Strains carrying heterologous plasmids were constructed as described above. For virulence assays, BALB/c mice were infected orally with a mixture of the wild-type strain and the same strain with an heterologous pSLT plasmid, and a CI was calculated for each combination. All the CI values obtained were around 1, indicating that the three plasmids make similar or identical contribution to virulence in their native backgrounds (Figure 4). This conclusion is consistent with the absence of nucleotide polymorphism in their *spv* regions (data not shown).

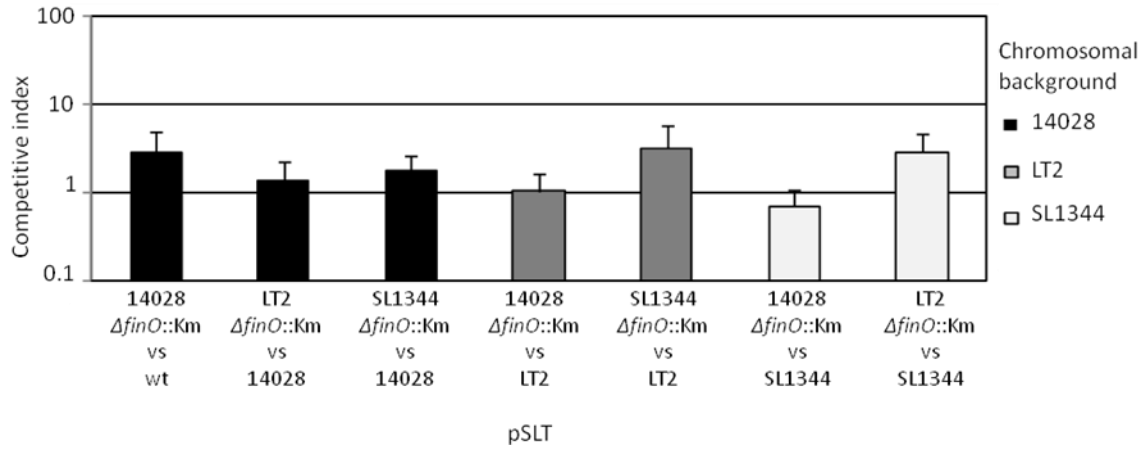


Figure 4. Competitive index analysis of virulence plasmids after oral infection of BALB/c mice. Mixed infections were as follows: SV4934/ATCC 14028; SV4478/ATCC 14028; SV4935/ATCC 14028; SV4934/LT2; SV4935/LT2; SV4934/SL1344; SV4478/SL1344. Histogram color codes indicate the chromosomal background: black, ATCC 14028; grey, LT2; white, SL1344. The CIs represented are averages from three infections. Error bars represent standard deviations.

DISCUSSION

Exchange of virulence plasmids between strains LT2, ATCC 14228 and SL1344 of *Salmonella enterica* serovar Typhimurium shows that each plasmid promotes conjugal transfer at a different frequency, regardless of the host background. Even though the virulence plasmid of strain SL1344 strain had been reported to be nonconjugative (Ahmer *et al.*, 1999), use of a FinO$^-$ derivative permits the detection of pSLT^{1344} transfer if matings are performed on minimal medium.

A change in a single nucleotide in the *traG* coding sequence, putatively causing an A $\rightarrow$ V amino acid substitution at position 940, seems to be the major cause of reduced transfer of pSLT^{1344}. Not surprisingly, the transfer-proficient pSLT^{LT2} and pSLT^{14028} plasmids are predicted to contain alanine at position 940, and alanine is also predicted to exist at the same position in the TraG products of *S.* Dublin pOU1113 and *E. coli* F (data not shown). Hence, the *traG* gene of pSLT^{1344} seems to have acquired a mutation that reduces conjugal transfer.

Allelic interchange of *traG* between pSLT^{LT2} and pSLT^{1344} increased and decreased 10 fold the mating frequency, respectively. Although these frequency changes are remarkable, introduction of an alanine codon in the *traG* gene did not increase transfer of pSLT^{1344} up to the level of pSLT^{LT2}. This observation indicates the existence of additional, unknown elements that contribute to reduced conjugal transfer of pSLT^{1344}.

We cannot ascertain whether divergence in the SL1344 virulence plasmid may have occurred in nature or after domestication. However, it is remarkable that the virulence plasmids of the three *Salmonella* model strains show divergence in the *tra* operon and not in the *spv* region. A tentative explanation might be that the virulence-required *spv* region is maintained by selection while conjugal transfer loci are allowed to undergo genetic drift. This hypothesis is supported by the fact that *Salmonella* virulence plasmids carrying incomplete *tra* operons are fairly common (Rotger & Casadesus, 1999). If these views are correct, pSLT^{1344} may be at an intermediate stage towards a non-conjugative form.

The ancestor of *Salmonella* subspecies I may have acquired the *spv* region by conjugation. Hence, the *tra* operon may have played a major role in the evolution of *Salmonella* by permitting acquisition of genetic determinants crucial for animal

infection. However, conjugation may have become neutral, if not deleterious, thereafter. Note that synthesis of the conjugation machinery and DNA transfer are energy-consuming processes that represent a burden for the bacterial cell (Frost & Koraimann, 2010). *Salmonella* Typhi does not contain a virulence plasmid (Boyd & Hartl, 1998). This view does not exclude, of course, that conjugal transfer of the virulence plasmid may have selective value under certain circumstances. For instance, *Salmonella* cells that lose their virulence plasmid in the duodenum as a consequence of bile-induced curing might have the opportunity to recover the plasmid in the ileum (Garcia-Quintanilla et al., 2006). On the other hand, even if conjugation has little or no selective value for the *Salmonella* host, it is a genuine mode of plasmid replication. Hence, the existence of *Salmonella* virulence plasmids that conserve conjugative capacity may be also viewed as a remnant of the ancestral tradeoff that permitted acquisition of the *spv* operon.

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DISCUSSION

1. Conjugation *in vivo*

Previous studies had shown that plasmids carrying antibiotic resistance determinants are spread *in vivo* by conjugal transfer within the same species or between different species (Lacy & Leary, 1975; Leverstein-van Hall *et al.*, 2002; Licht *et al.*, 1999; Salyers *et al.*, 2004; Schjorring *et al.*, 2008). For a conjugative plasmid, conjugal transfer is a mode of replication and thus can be expected to have selective advantage. Plasmid pSLT carries a functional FinOP system of fertility inhibition and promotes mating at low frequencies *in vitro* (Camacho & Casadesus, 2002; Smith *et al.*, 1973; Torreblanca *et al.*, 1999), especially if compared with the F sex factor which is derepressed for transfer (Yoshioka *et al.*, 1987). However, transfer of pSLT is under additional controls by host-encoded functions that may adjust mating to favorable circumstances. The list includes a metabolism-related regulator, Lrp (Camacho & Casadesus, 2002), an oxygen-sensing signal transduction system, ArcAB (Serna *et al.*, 2010; Silverman *et al.*, 1991), as well as a regulator of carbon metabolism, FruR (Cra), and the master regulator of flagella synthesis (FlhDC) (A. Serna and J. Casadesús, unpublished). The existence of so many controls thus raises the possibility that transfer of pSLT in natural environments may follow rules different from those observed in the laboratory.

A relevant environment in *Salmonella* lifestyle is the mammalian gut. Hence, it is not surprising that plasmid pSLT can promote conjugation in the ileum of mouse. Because the population sizes and cell densities attained by *Salmonella* in the small intestine (Ohl & Miller, 2001) are unlikely to be found in other environments, intestinal mating may provide an appropriate environment for plasmid spread.

The pH in gut varies between 6 and 7 (Argenzio & Southworth, 1975; Bohnhoff *et al.*, 1964; Cummings *et al.*, 1987) and the lumen of the distal small intestine is microaerobic (Backhed *et al.*, 2005). The frequency of conjugation *in vitro* is always higher in solid than in liquid medium, which is logical if we assume that disruption of cell-to-cell contact is less likely in a static medium. Moreover, mating in nature has been reported to take place inside biofilms (Beaudoin DL, 1998; Christensen *et al.*, 1998; Ghigo, 2001; Hausner & Wuertz, 1999; Licht *et al.*, 1999; Piper & Farrand, 1999; Roberts *et al.*, 2001; Wang *et al.*, 2002). In the case of *Salmonella*, it is conceivable that mating may occur in the mucus layer of the intestine and the possibility of biofilm formation cannot be excluded.

The gut provides a rich supply of nutrients which permit rapid multiplication of *Salmonella* cells. Nutrient gradients probably provide significant signals to intestinal bacteria. For example, dietary glucose is rapidly absorbed in the upper intestine of all mammals. The concentration of glucose drops from ~25 mM in the upper duodenum to 1 mM in the mid-intestine and is barely detectable in the distal small intestine (Ferraris *et al.*, 1990). Many bacterial fimbrial operons are regulated in response to glucose. Synthesis of fimbriae by *V. cholerae* and enterotoxigenic *E. coli* is induced by growth on carbon sources such as glucose (Puente *et al.*, 1996; Skorupski & Taylor, 1997), and the bacteria adhere to the upper small intestine. Conversely, expression of other fimbriae that mediate attachment to the distal small intestine is enhanced by growth on poor carbon sources (Edwards & Schifferli, 1997; Lo-Tseng *et al.*, 1997). In *Salmonella*, Mlc represses several genes involved in sugar uptake and metabolism, and exerts positive effects on the expression of SPI-1 genes (Lim *et al.*, 2007). Similarly, FruR represses genes that are active in catabolism (Saier & Ramseier, 1996) and represses *traJ* transcription, thus inhibiting conjugation (Serna, A. and Casadesús, J., unpublished data). Therefore, it is conceivable that glucose can be used as a niche indicator. LB medium, which is a rich medium with a relatively poor glucose content, may in turn mimic (to a certain extent) the distal portion of the small intestine.

Bile salts have also a niche indicator role. Bile enhances bacterial co-aggregation, bacterial-intestinal epithelial cell adhesion and biofilm formation in *Bacteroides fragilis*, which is rarely found in the human duodenum but relatively frequent in gall bladder infections (Pumbwe *et al.*, 2007). Bile also induces the formation of *Salmonella* biofilms on the surface of gallstones during chronic *Salmonella* infection (Prouty *et al.*, 2003) and down-regulates secretion and invasion genes of pathogenicity island 1 (Prouty & Gunn, 2000). Because of its detergent activity, bile salts can permeabilize bacterial membranes and eventually lead to membrane collapse and cell damage (Begley *et al.*, 2005; Bron *et al.*, 2004; De Boever & Verstraete, 1999). Tra pili encoded by pSLT do not cause bile sensitivity as they do in F (Bidlack & Silverman, 2004). However, it is conceivable that cell-to-cell interactions can be disturbed by the presence of bile salts, resulting in diminished ability to conjugate.

We have also observed that bile has a curing effect on the *Salmonella* virulence plasmid. In the past, the anionic detergent sodium dodecyl sulphate (SDS) was known to cure certain plasmids which synthesized pili constitutively (Inuzuka

et al., 1969; Tomoeda *et al.*, 1968). There is some controversy about this point. Some authors propose that exposure to SDS selected non-piliated cells, simply because piliated *E. coli* cells were more susceptible to lysis by SDS. Hence, the increase in the proportion of non-piliated cells was not caused by a direct action on the plasmid; it simply reflected the selective advantage enjoyed by spontaneous non-piliated variants (Salisbury *et al.*, 1972). Other authors, however, sustain the hypothesis that SDS eliminates F and R plasmids (Krivoshein Yu *et al.*, 1988). Whatever the case, these results are unlikely to apply to the pSLT-encoded pilus because it is resistant to bile. Hence, we propose that bile cures pSLT as a consequence of the DNA-damaging activity of bile salts, which causes DNA rearrangements and point mutations that can impair DNA replication (Prieto *et al.*, 2004). In fact, many DNA-damaging agents cause plasmid curing (Willetts, 1967).

The epithelium of the proximal small intestine consists mainly of villous epithelial cells, interspersed with specialized M cells, which are clustered in Peyer's patches. In contrast, in the epithelium of the distal small intestine the M cells are more abundant (Siebers & Finlay, 1996). *Salmonella* infects preferentially M cells in the ileum, and the conditions used *in vitro* to induce SPI-1 (rich medium and microaerobiosis) (Figure D.1) are supposed to mimic the natural environment that *Salmonella* finds in the distal small intestine (Eichelberg & Galan, 1999). These are exactly the conditions that we have used to test conjugation in the laboratory.

The large intestine contains a large number of indigenous microorganisms. This characteristic limits the availability of suitable niches for opportunistic pathogens (Batt *et al.*, 1996); consequently, few pathogens have been characterized as causing disease in the large intestine. Propionate is produced by the anaerobic microbiota inhibiting growth of *Salmonella* (Bohnhoff *et al.*, 1964; Meynell, 1963) and decreasing invasion gene expression (Lawhon *et al.*, 2002). Sulfide is produced by sulfate-reducing bacteria of flora (Shoveller *et al.*, 2005) and both propionate and sulfide inhibit pSLT transfer (Figure D.2). A tentative interpretation is that inhibition of conjugation in the large intestine may prevent a waste of energy when *Salmonella* competes for nutrients with the bacterial flora. Moreover, the presence of these compounds may indicate that *Salmonella* has left the ileum, making *spv* genes (and thus plasmid spread) no longer advantageous.

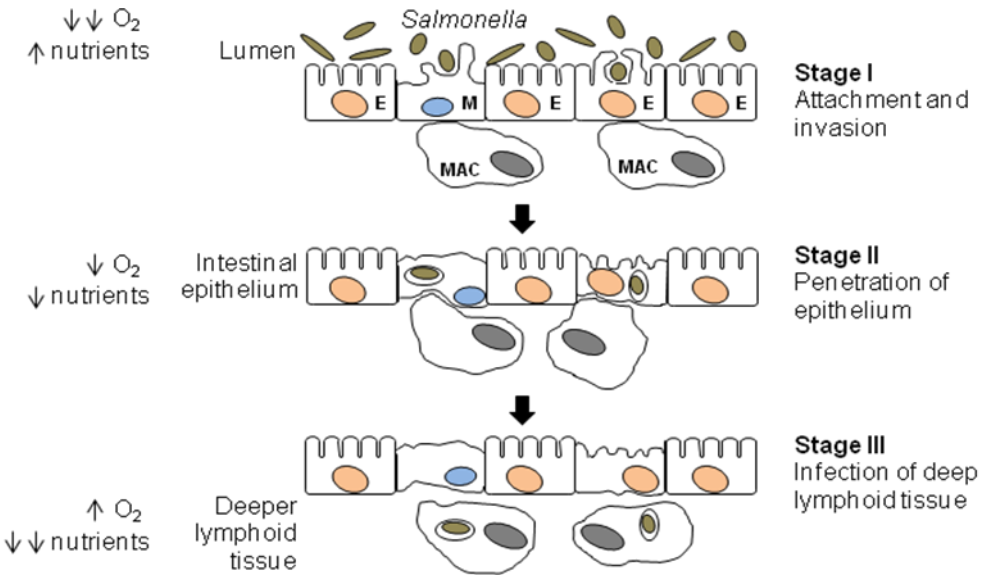

FIGURE D.1 Diagram for sequential invasion steps of *Salmonella* after oral infection. In stage I, *Salmonella* is present in the lumen of the small bowel, an environment that is characterized by a low level of oxygen and a modest nutrient supply. These conditions may favor the expression of the invasion (*inv*) and conjugation (*tra*) genes. In stage II, *Salmonella* within epithelial cells (E) encounters a combination of decreased nutrients in the intracellular vacuole and increased oxygenation. In stage III, M cells (M) and probably other epithelial cells are destroyed by the bacteria as they pass into deeper tissues. Bacteria disseminating from the gastrointestinal tract are phagocytosed by macrophages (MAC) and encounter conditions that may induce *spv* and downregulate SPI-1. [Adapted from Guiney et *al* (Guiney *et al.*, 1995)].

We may assume that the control of conjugation along the intestinal tract might be similar to that shown by other factors involved in intestinal processes, such as fimbriae and SPI-1, whose expression depends on environmental signals. On the other hand, *Salmonella* colonization of animals requires competition with the established intestinal flora. For this reason, treatment of mice with antibiotics prior to *Salmonella* administration is an old practice used to boost experimental *Salmonella* infections (Bohnhoff *et al.*, 1964). However, we have detected high frequencies of conjugal transfer without antibiotic pretreatment of mice (in other words, under circumstances that do not especially favor *Salmonella* proliferation). Altogether, these considerations suggest that intestinal transfer of virulence plasmids may indeed occur in house mice, and perhaps in other animals, during *Salmonella* infection.

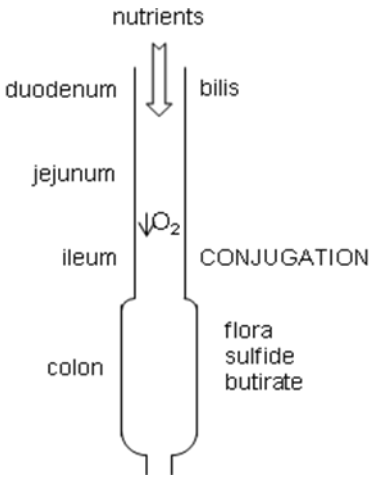

FIGURE D.2 Representation of gut conditions which affect mating frequency. Duodenum is characterized by having the highest concentration of bile, which inhibits mating. Large intestine contains a large number of indigenous microorganisms that produce sulfide and butirate, among other compounds, that decrease conjugation.

2. FinP-mediated control of L-cysteine biosynthesis and the role of L-cysteine as an inhibitor of mating

In contrast with the numerous examples of bacteriophage interference with host gene expression (Boeckh *et al.*, 1986; Bunny *et al.*, 2002; Duckworth, 1970; Nechaev & Severinov, 2003), the ability of plasmids to control host gene expression has few precedents, except for the activities of addiction modules that target host functions upon plasmid loss (Zielenkiewicz & Ceglowski, 2001).

We provide preliminary evidence that FinP RNA encoded in the virulent plasmid of *Salmonella enterica* may work both as a *cis*-acting antisense RNA and as a *trans*-acting small regulatory RNA. Expression of the host-encoded *cysDNC* operon increases with the overproduction of FinP RNA. The underlying molecular mechanisms remain to be clarified, many alternative models can be proposed. FinP may directly interact with the *cysDNC* transcript, enhancing its stability. This possibiliy is supported by the observation that FinP RNA does not increase the transcripts of *cysB*, which encodes a positive transcriptional regulator of the *cysDNC* operon. However, it remains possible that FinP RNA might control the stability and/or the activity of the CysB protein. Albeit less known than standard, small RNAs that target other RNA molecules, small regulatory RNAs that exert their control upon proteins have been described in the literature (Lapouge *et al.*, 2008; Lenz *et al.*, 2005; Romeo, 1998; Wassarman & Storz, 2000). On the other hand,

evidence exists that no other element from the plasmid, aside from FinP, is involved in the *cysDNC* regulation: increased levels of *cysDNC* mRNA are found in a pSLT-cured strain that expresses FinP RNA under the control of an heterologous P_{BAD} promoter (M. García-Quintanilla, K. Papenfort, J. Vogel and J. Casadesús, unpublished). The possible drawback of FinO absence in a cured strain is unlikely to pose a problem because high levels of FinP usually alleviate the need for FinO (Koraimann *et al.*, 1991; Koraimann *et al.*, 1996). Moreover, it is not advisable to clone *finO* on a high copy number plasmid because it may cause growth problems (Tim van Biesen and Laura S. Frost, unpublished observation from the paper (van Biesen & Frost, 1994)).

Genetic analysis provides unsuspected evidence that L-cysteine metabolism affects conjugal transfer: *cysD* as well as *cysI asrC* donors of *S.* Typhimurium showed a 10 fold increase in the frequency of conjugation. These results suggest that ATP sulfurylase (CysDN) does not have a secondary activity involved in conjugation, and that the activity of the sulfate reduction pathway is involved indeed in the plasmid transfer phenotype. The involvement of sulfur metabolism in mating control is additionally supported by the following observations: i) L-cysteine and sulfide reduce conjugal transfer, and ii) L-cysteine inhibits conjugation in the wild type as well as in a *cysD* donor, suggesting that in a *cysD* donor the lower concentrarion of the final products of the pathway may cause increased mating.

As indicated above, sulfide is produced by the microbiota of the large intestine, using oxidized sulfur compounds present in food as well as endogenous sulfated mucins (Shoveller *et al.*, 2005). Sulfur-containing compounds might serve as signals in specific environments to inhibit conjugation. In the case of sulfide, it may inform *Salmonella* that the milieu is the large intestine, where conditions for mating are not optimal. L-cysteine has been reported to perform signaling roles in other microorganisms. In *Bordetella pertussis* the availability of cysteine regulates toxin synthesis through the metabolic conversion of cysteine to SO_4^{2-} and pyruvic acid (Bogdan *et al.*, 2001). In *Salmonella*, however, we can assume that the effect of L-cysteine on conjugation depends on L-cysteine itself and not on SO_4^{2-} since when the sulfate reduction pathway is interrupted and L-cysteine is added, L-cysteine remains an inhibitor.

Cystine supplementation in mice has been shown to reduce the intestinal translocation of *Salmonella enterica* serovar Enteritidis likely due to the

enhancement of gut barrier functions (van Ampting *et al.*, 2009). This observation is in accordance with the idea that virulence, adhesion and conjugation in *Salmonella* respond to signals that let sense the environments along the intestine. A general concept could be that L-cysteine is related to immune response of mammals and *Salmonella* might avoid invasion and conjugation in the presence of high concentrations of cysteine because type IV secretion systems might be immunogenic (Smith & Huggins, 1975). An alternative possibility is that the sulfate reduction pathway of *S.* Typhimurium might be activated in response to toxic oxidants as described for *Mycobacterium tuberculosis* (Pinto *et al.*, 2004), and FinP might contribute to inhibit conjugation under such circumstances. The oxidative damage caused by bile salts might activate the sulfate reduction pathway and *cysDNC* enhanced expression might contribute to produce inhibitory signals for conjugation in the duodenum.

In *E. coli* L-cysteine has been suggested to be a signal molecule that inhibits biofilm development (Sturgill *et al.*, 2004). In fact, *cysB* and *cysE* mutants form biofilm at an accelerate rate (Ren *et al.*, 2005; Sturgill *et al.*, 2004). On the other hand, *cysB* and *cysE* mutants of *S.* Typhimurium confer resistance to mecillinam which is abolished by supplementation of thiosulfate, sulfite, sulfide (precursors of cysteine) and cysteine (Oppezzo & Anton, 1995). The phenotype of resistance could be directly related with biofilm formation since it is known that cells in biofilms are more resistant to antibacterial agents than their planktonic counterparts (Brown *et al.*, 1988; Mah & O'Toole, 2001; Stewart, 1996). L-cysteine might thus decrease resistance due to inhibition of biofilm formation.

A recent study has shown that *S.* Typhimurium *cysB* and *cysE* mutants fail to swarm unless cysteine is supplemented to the medium (Turnbull & Surette, 2008). Swarming and biofilm formation are mutually exclusive in *S. enterica* (Harshey, 2003; Mireles *et al.*, 2001), and evidence exists that biofilm formation may favour mating in natural environments (Ghigo, 2001; Hausner & Wuertz, 1999; Licht *et al.*, 1999; Piper & Farrand, 1999) presumably by increasing high cell density and especially by maintaining the bacterial cells in close proximity. Hence, L-cysteine might act as a switching molecule whose presence might favor swarming motility and prevent conjugal transfer. This is a reasonable scenario since higher frequencies of conjugation might take place between motionless bacteria. In fact, the major activator of the flagellum synthesis, FlhDC, downregulates conjugation via repression of *traJ* (Serna, A. and Casadesús, J., unpublished data).

There is only another example in *E. coli* of a plasmid-encoded RNA that controls the expression of chromosomal genes (Chant & Summers, 2007). Multicopy plasmid ColE1 encodes an untraslated RNA of 70 nucleotides called Rcd (regulator of cell division) (Balding *et al.*, 2006; Patient & Summers, 1993; Sharpe *et al.*, 1999). Rcd binds chromosome-encoded tryptophanase and increases its affinity for the substrate tryptophan, producing high levels of indole which acts as a signal molecule and delays cell division until plasmid multimers have been resolved to monomers (Chant & Summers, 2007). However, FinP is the first example of an antisense RNA that may additionally act as a small regulatory RNA. Regulation of cysteine biosynthesis by FinP RNA thus shows that the FinOP system of fertility inhibition is more complex and sophisticated than previously envisaged, since it involves not only downregulation of *traJ* mRNA but also FinP-mediated upregulation of a chromosomal transcript (cysDNC) whose products contribute to mating inhibition (Figure D.3). Regulation of conjugal transfer in the *Salmonella* virulence plasmid, and potentially in other F-like plasmids, may therefore involve bidirectional crosstalk between the plasmid and the host genome.

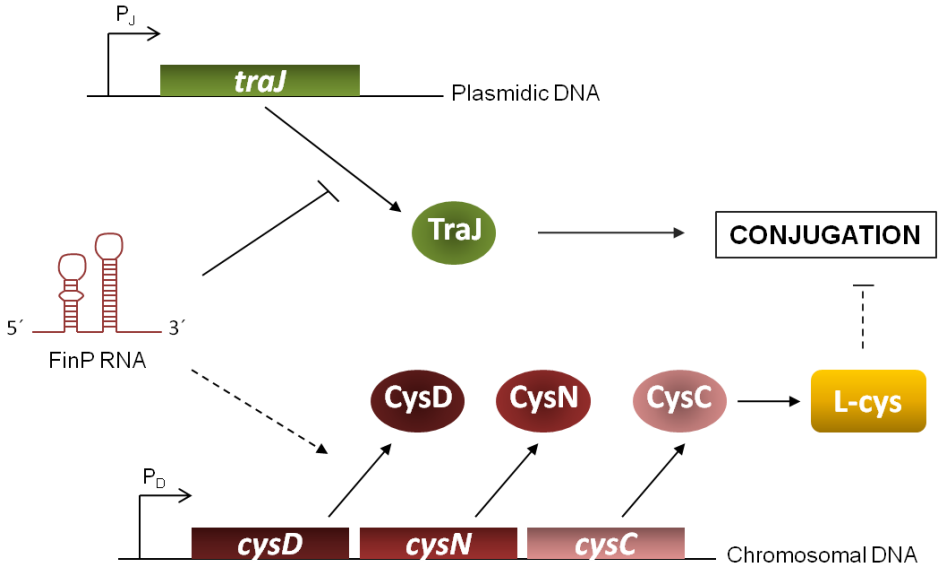

FIGURE D.3 Model for conjugal transfer regulation by FinP as a cross-talk molecule between pSLT and the chromosomal DNA. FinP inhibits conjugation through two different ways: i) as antisense of *traJ*, FinP prevents the translation of the main activator of *tra* operon; ii) as small RNA *in trans*, FinP is able of stabilizing *cysDNC* mRNA, direct or indirectly, with the ulterior increment of cysteine, which inhibits conjugation. Arrows indicate activation and blunt lines denote inhibition. Solid lines indicate direct interaction, whereas dotted lines indicate indirect or unknown interaction.

3. Is conjugal transfer ability a neutral trait for *Salmonella*?

Although environmental adaptation by internal genome dynamics does occur and has been documented both in natural populations (Sokurenko *et al.*, 1998) and in the laboratory (Papadopoulos *et al.*, 1999; Rainey & Travisano, 1998), lateral gene transfer allows fully functional pathways to be acquired and used for efficient exploitation of novel environments (Frost *et al.*, 2005). Microbial species are created by continual processes of gene loss and acquisition promoted by horizontal genetic transfer (Groisman & Ochman, 1994).

The best known traits of the *Salmonella enterica* serovar Typhimurium plasmid are its involvement in virulence and its ability to promote conjugation (Ahmer *et al.*, 1999; Gulig & Curtiss, 1987). Many laterally acquired elements like plasmids, prophages or transposable elements normally carry functions that have some benefit for their hosts, such as antibiotic resistance or virulence related genes. However, these elements can also have characteristics typical of "selfish" genetic elements. One is the promotion of self-spread (host-independent reproduction). Another is the capacity to kill the hosts that lose them. In the case of pSLT, spread is achieved by conjugation, perhaps as a trade-off that enables *Salmonella* populations to become more virulent. In turn, a functional toxin-antitoxin *ccdAB* system prevents plasmid loss in the bacterial population.

Once DNA has been laterally acquired, it begins a co-evolutive and adaptive process between host and guest, which can end in assimilation or loss of the foreign DNA. It has been proposed that the nucleoid protein H-NS could inhibit expression of newly acquired DNA (Lucchini *et al.*, 2006). H-NS principally binds DNA with high AT content. In *S.* Typhimurium, H-NS regulates about 42% of laterally acquired genes, including pathogenicity islands and the *tra* operon of pSLT (Garcia-Vallve *et al.*, 2003). Co-evolution is specific for each host and each guest. For instance, our results show that the type IV secretion apparatus encoded on pSLT seems to have evolved in a different manner from its homolog in the *E. coli* F plasmid, which sensitizes *E. coli* to bile salts (Bidlack & Silverman, 2004). In contrast, Salmonellae expressing pSLT-encoded pili are bile-resistant. The relatedness between the transfer proteins of F and pSLT is low: the amino acid identities are only 68% for TraV, 33% for TraP, 29% for TraY, and 14% for TraS. In turn, comparisons of the serovar-specific plasmids of *Salmonella enterica* point clear differences from one plasmid to another. Each virulence plasmid seems to be

specific to its host, as exemplified by the fact that plasmid size is more or less strain-specific. All plasmids keep the *spv* locus (Gulig *et al.*, 1993); in contrast, the *tra* locus remains intact only in *S.* Typhimurium, as its virulence plasmid is still capable of conjugation (Ahmer *et al.*, 1999). The virulence plasmids of *S.* Gallinarum/Pullorum and *S.* Enteritidis are still capable of mobilization by the F plasmid whereas the plasmids of *S.* Dublin and *S.* Choleraesuis are not mobilizable due to *oriT* absence (Barrow & Lovell, 1989; Chu *et al.*, 2002; Ou *et al.*, 1994). In these serotypes, the *tra* locus has been subjected to considerable evolutionary erosion leading to loss of conjugative transfer or mobilization. These observations suggest that the *spv* operon was horizontally transmitted by conjugation to a *Salmonella* ancestor. However, in contrast with its important role in *Salmonella* evolutionary history, conjugal transfer may have become superfluous. Hence, it is not suprising that the virulence plasmids of strains ATCC 14028, LT2, and SL1344 have intrinsically different mating frequencies while they are interchangeable for virulence.

Even though the virulence plasmid of strain SL1344 strain had been reported to be nonconjugative (Ahmer et al., 1999), use of a FinO$^-$ derivative permits the detection of pSLT^{1344} transfer if matings are performed on minimal medium. A change in a single nucleotide in the *traG* coding sequence, putatively causing an A $\rightarrow$ V amino acid substitution at position 940, seems to be the major cause of reduced transfer of pSLT^{1344}. Not surprisingly, the transfer-proficient pSLT^{LT2} and pSLT^{14028} plasmids are predicted to contain alanine at position 940, and alanine is also predicted to exist at the same position in the TraG products of *S.* Dublin pOU1113 and *E. coli* F (data not shown). Hence, the *traG* gene of pSLT^{1344} seems to have acquired a mutation that reduces conjugal transfer.

Allelic interchange of *traG* between pSLT^{LT2} and pSLT^{1344} increased and decreased 10 fold the mating frequency, respectively. Although these frequency changes are remarkable, introduction of an alanine codon in the *traG* gene did not increase transfer of pSLT^{1344} up to the level of pSLT^{LT2}. This observation indicates the existence of additional, unknown elements that contribute to reduced conjugal transfer of pSLT^{1344}.

We cannot ascertain whether divergence in the SL1344 virulence plasmid may have occurred in nature or after domestication. However, it is remarkable that the virulence plasmids of the three *Salmonella* model strains show divergence in the *tra* operon and not in the *spv* region. A tentative explanation might be that the virulence-required *spv* region is maintained by selection while conjugal transfer loci are allowed to undergo genetic drift. This hypothesis is supported by the fact that *Salmonella* virulence plasmids carrying incomplete *tra* operons are fairly common (Rotger & Casadesus, 1999). If these views are correct, pSLT^{1344} may be at an intermediate stage towards a non-conjugative form.

The ancestor of *Salmonella* subspecies I may have acquired the *spv* region by conjugation. Hence, the *tra* operon may have played a major role in the evolution of *Salmonella* by permitting acquisition of genetic determinants crucial for animal infection. However, conjugation may have become neutral, if not deleterious, thereafter. Note that synthesis of the conjugation machinery and DNA transfer are energy-consuming processes that represent a burden for the bacterial cell (Frost & Koraimann, 2010). *Salmonella* Typhi, which carries the *spv* region on the chromosome, does not contain a virulence plasmid (Boyd & Hartl, 1998). This view does not exclude, of course, that conjugal transfer of the virulence plasmid may have selective value under certain circumstances. For instance, *Salmonella* cells that lose their virulence plasmid in the duodenum as a consequence of bile-induced curing might have the opportunity to recover the plasmid in the ileum. On the other hand, even if conjugation has little or no selective value for the *Salmonella* host, it is a genuine mode of plasmid replication. Hence, the existence of *Salmonella* virulence plasmids that conserve conjugative capacity may be also viewed as a remnant of the ancestral tradeoff that permitted acquisition of the *spv* operon.

CONCLUSIONS

1. The virulence plasmid of *Salmonella enterica* serovar Typhimurium contains a functional addiction module, homologous to the CcdAB module of the F sex factor. The pSLT CcdAB module reduces curing of the virulence plasmid in the presence of bile.

2. The virulence plasmid of *Salmonella enterica* serovar Typhimurium is transferred at high rates in the gut of mice.

3. Microaerobiosis activates conjugal transfer of the *Salmonella* virulence plasmid under laboratory conditions.

4. Sodium propionate inhibits conjugal transfer of the *Salmonella* virulence plasmid under laboratory conditions.

5. FinP RNA encoded by the *Salmonella* virulence plasmid increases the expression of the *cysDNC* chromosomal operon. Increased expression may reflect increased mRNA stability in the presence of FinP RNA.

6. L-cysteine, and to a lesser extent sodium sulfide, are inhibitors of conjugal transfer of the pSLT plasmid.

7. The virulence plasmid of model strain SL1344 promotes conjugal transfer at reduced frequencies, compared with the plasmids of LT2 and ATCC 14028. An A $\rightarrow$ V amino acid substitution in the TraG protein appears to be the major cause that reduces conjugal transfer in SL1344.

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