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Étude fonctionnelle de l'opéron fimbriaire *stg* de *Salmonella enterica* sérovar Typhi

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Étude fonctionnelle de l'opéron fimbriaire *stg* de *Salmonella enterica* sérovar Typhi

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Résumé

La bactérie *Salmonella enterica* sérovar Typhi (*S.* Typhi) provoque la fièvre typhoïde chez les humains et constitue un problème de santé publique important. La majorité de nos connaissances sur la pathogenèse de cette bactérie provient du modèle de fièvre entérique chez la souris causée par le sérovar Typhimurium. Peu d'études se sont penchées sur les facteurs de virulence uniques au sérovar Typhi, ni sur la possibilité que les pseudogènes retrouvés dans son génome puissent être fonctionnels. Le fimbria *stg*, unique au sérovar Typhi, renferme un codon d'arrêt TAA prématuré dans le gène *stgC* qui code pour le placier responsable de l'assemblage des sous-unités fimbriaires à la surface de la bactérie. Ainsi, le fimbria *stg* a été classifié dans la liste des pseudogènes non-fonctionnels. Les objectifs de cette étude étaient d'évaluer l'implication du fimbria *stg* lors de l'interaction avec les cellules humaines, puis de vérifier l'importance du pseudogène *stgC* lors de la biogenèse fimbriaire.

Dans une première partie, la transcription de *stg* a été évaluée à l'aide d'une fusion *lacZ*. Malgré des niveaux d'expression observés généralement faibles en milieu riche, la croissance en milieu minimal a favorisé la transcription de l'opéron. La délétion complète de l'opéron fimbriaire *stgABCD* du génome de *S.* Typhi a été réalisée par échange allélique, puis a été complémentée sur un plasmide. Il a été démontré que la présence de *stg* chez *S.* Typhi, *S.* Typhimurium et *E. coli* contribue à une adhérence accrue sur les cellules épithéliales humaines. De plus, ce fimbria semble agir comme une structure anti-phagocytaire lors de l'interaction avec des macrophages humains. Ainsi, l'opéron *stg* semble fonctionnel, malgré son codon d'arrêt prématuré, puisque des phénotypes ont été observés.

La seconde partie de cette étude consistait à vérifier le rôle joué par le pseudogène *stgC* dans la biogenèse du fimbria. Différentes variantes de l'opéron ont été générées, clonées dans un vecteur inductible à l'arabinose, puis transformées dans la souche

afimbriaire d'*E. coli* ORN172. La translocation de la sous-unité fimbriaire StgD à la surface de la bactérie a été évaluée chez ces différents mutants par immunobuvardage de type Western. Cette expérience a permis de démontrer que le pseudogène *stgC* est essentiel pour l'exportation de la sous-unité StgD à la surface. L'ajout d'une étiquette de 6-histidines en C-terminal de StgC a permis de confirmer la traduction complète du gène, malgré le codon d'arrêt TAA prématuré. Le séquençage peptidique a révélé l'insertion d'une tyrosine à ce codon. Une fusion traductionnelle avec la protéine verte fluorescente a révélé qu'environ 0.8% de l'ARNm peut être traduit et permet la production complète du placier.

Ce projet a permis la caractérisation d'un facteur de virulence unique à *S.* Typhi et constitue une étape de plus vers la compréhension de ses mécanismes de pathogenèse. Il s'agit de la première démonstration chez les bactéries de la fonctionnalité d'un gène interrompu prématurément par un codon d'arrêt TAA.

Mots-clés : *Salmonella enterica* sérovar Typhi, fimbriae, Stg, pathogenèse, pseudogène

Abstract

Salmonella enterica serovar Typhi (*S*. Typhi) causes typhoid fever in humans and is considered as an important health problem. Most of our knowledge on the pathogenesis of this bacterium comes from an enteric fever model in mice caused by serovar Typhimurium. Few studies have examined the virulence factors unique to serovar Typhi or the possibility that pseudogenes harbored in its genome may be functional. *stg* fimbriae are found only within the serovar Typhi genome and contain a premature TAA stop codon in the *stgC* gene encoding the usher responsible for the assembly of fimbrial subunits at the bacterial surface. Thus, the *stg* fimbria has been classified among the list of non-functional pseudogenes. The objectives of this study were to assess the involvement of *stg* fimbriae during interaction with human cells, and then to evaluate the importance of the *stgC* pseudogene in fimbrial biogenesis.

First, *stg* transcription was evaluated using a *lacZ* fusion. Despite low expression levels generally observed in rich medium, growth in minimal medium promoted transcription of the operon. Complete deletion of the *stgABCD* fimbrial operon from *S*. Typhi was performed by allelic exchange and was complemented on a plasmid. It has been shown that the presence of *stg* in *S*. Typhi, *S*. Typhimurium and *E. coli* contributes to increased adherence to human epithelial cells. In addition, the fimbriae seem to act as an anti-phagocytic structure during the interaction with macrophages. Thus, the *stg* operon appears to be functional despite its premature codon, as phenotypes were observed.

The second part of this study involved testing the role of the *stgC* pseudogene in fimbrial biogenesis. Different variants of the operon were generated, cloned into an arabinose inducible vector, and then transformed into afimbriated *E. coli* strain ORN172. Translocation of the StgD subunit to the cell surface of the different mutants was evaluated using Western blot. This experiment demonstrated that *stgC* is essential for export of the StgD subunit to the cell surface. The addition of a 6-histidine tag at the C-terminal end of

StgC confirmed the complete translation of the gene, despite the premature TAA stop codon. Peptide sequencing revealed the insertion of a tyrosine at this codon. A translational fusion with the green fluorescent protein demonstrated that approximately 0.8% of the mRNA can be translated to allow full production of the usher.

This project allowed characterization of a virulence factor unique to *S.* Typhi and is a step closer towards better understanding of its pathogenesis mechanisms. This is the first demonstration in bacteria of the functionality of a gene which is interrupted by a premature TAA stop codon.

Keywords : *Salmonella enterica* serovar Typhi, fimbriae, Stg, pathogenesis, pseudogene

Table des matières

Liste des tableaux

CHAPITRE 1 :

CHAPITRE 2

CHAPITRE 3 :

Liste des figures

CHAPITRE 1 :

Section 1.4

CHAPITRE 2:

CHAPITRE 3:

CHAPITRE 4:

ANNEXE I:

ANNEXE II:

Liste des sigles et abréviations

A. A.	Acide aminé
ADN	Acide désoxyribonucléique
Ap	Ampicilline
ARN	Acide ribonucléique
ARNm	ARN messager
ARNt	ARN de transfert
ATP	Adénosine triphosphate
CFTR	*Cystic Fibrosis Transmembrane Conductance Regulator*
CFU	*Colony-Forming Unit*
DAP	*Diaminopimelic Acid*
GFP	*Green fluorescent protein*
H-NS	*Histone-like Nucleoid Structuring protein*
IHF	*Integration Host Factor*
IPTG	Isopropyl-bêta-thiogalactopyranoside
kb	Kilobase
LB	Luria-Bertani
LPS	Lipopolysaccharide
Mb	Mégabase
MDR	*Multi-drug resistant*
ORF	*Open Reading Frame*
PMA	M phorbol 12-myristate 13-acetate
SPI	*Salmonella* Pathogenicity Island
SST1	Système de sécrétion de type 1
SST3	Système de sécrétion de type 3
SST5	Système de sécrétion de type 5
S. Typhi	*Salmonella enterica* subsp. *enterica* sérovar Typhi
S. Typhimurium	*Salmonella enterica* subsp. *enterica* sérovar Typhimutium

Il n'y a point de génie sans un grain de folie

Aristote, 384-322 av. J.-C.

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CHAPITRE 1 – Revue de la littérature

1.1. Généralités sur *Salmonella*

Les salmonelles sont des bactéries communément retrouvées chez une grande variété d'animaux, chez l'humain et parfois dans l'environnement (231). Elles peuvent être associées avec les intestins de leurs hôtes de façon asymptomatique ou causer des infections telles la gastro-entérite ou la fièvre entérique.

La découverte de *Salmonella enterica* subsp. *enterica* sérovar Typhi a été créditée à Karl Joseph Eberth qui a observé en 1880 des organismes en forme de bâtonnets provenant de la rate et des ganglions de patients atteints de typhoïde (62). Cependant, le nom *Salmonella* a été suggéré en 1900 pour honorer le laboratoire du vétérinaire Daniel Elmer Salmon où l'isolation de *S*. Choleraesuis d'intestins de porcs a été réalisée (20).

Les salmonelles sont des bâtonnets à Gram négatif, anaérobies facultatifs et faisant partie de la famille des *Enterobacteriaceae* (16). *Salmonella* peut croître à des températures situées entre 6-47°C, mais fait partie des bactéries mésophiles ayant une température de croissance optimale à 37°C (201). C'est une bactérie neutrophile puisque sa croissance est optimale dans un pH entre 6.5-7.5, mais elle peut tout de même croître à pH 4.05-9.5 (62). La majorité des sérovars sont prototrophes, mais certains sérovars adaptés à un hôte, comme *S*. Typhi, peuvent être auxotrophes (62). Ce qui caractérise principalement le genre *Salmonella* est l'impressionnante diversification des niches écologiques chez les différents sérovars malgré un haut degré d'homologie génétique (199).

1.1.1. Nomenclature et taxonomie

La nomenclature de *Salmonella* s'avère particulièrement complexe et évolue constamment. Les différentes « espèces » ont d'abord été nommées selon les symptômes observés lors de la maladie, puis selon leur lieu géographique d'isolation (21). Le schéma de Kauffmann-White classifie les différents sérovars selon leurs antigènes de surface et constitue un outil encore utilisé à ce jour pour les nouveaux isolats non-caractérisés (115, 193). La formule antigénique d'un sérovar (ou sérotype) est définie comme suit : l'antigène O (partie exposée du LPS), suivi de la capsule Vi (si présente), puis de l'antigène H (flagelle de phase 1, phase 2 si présent) (21). Aujourd'hui, le genre *Salmonella* renferme officiellement 2 espèces, soit *S. bongori* et *S. enterica* (21). Cependant, l'isolation récente d'une bactérie prélevée de sédiments acides de l'environnement suggèrerait la possibilité d'une troisième espèce désignée *S. subterranea* (231). L'espèce *S. enterica* renferme plus de 2500 sérovars qui sont distribués parmi 6 sous-espèces (voir Tableau I) (198). En théorie, chaque espèce devrait être désignée par cette longue appellation : *Salmonella enterica* subsp. *enterica* sérovar Typhi. Afin d'alléger cette thèse, seuls le genre et le sérovar seront utilisés comme suit : *S.* Typhi.

Tableau I. Espèces retrouvées chez *Salmonella*, nomenclatures utilisées et nombre de sérovars répertoriés en 2002, adapté de (198, 239).

Espèces	Nomenclature d'origine	Nomenclature actuelle	Nombre de sérovars répertoriés
S. enterica	subsp. I	subsp. *enterica*	1504
	subsp. II	subsp. *salamae*	502
	subsp. IIIa	subsp. *arizonae*	95
	subsp. IIIb	subsp. *diarizonae*	333
	subsp. IV	subsp. *houtenae*	72
	subsp. VI	subsp. *indica*	13
S. bongori	subsp. V		22
S. subterranea			

Subsp. signifie sous-espèce

1.1.2. Évolution à partir des entérobactéries

Plusieurs évènements d'acquisition de gènes par transferts horizontaux et de recombinaisons génétiques semblent être la cause de l'apparition et de la diversification du genre *Salmonella* de celui d'*Escherichia coli*. L'absence de séquençage génomique de *S. subterranea* ne permet pas de l'inclure pour le moment dans l'arbre phylogénétique des salmonelles (Fig. 1.1).

L'apparition des mammifères il y a plus de 120 millions d'années correspondrait à la diversification du genre *Salmonella* de celui d'*Escherichia coli* (175). Ces deux bactéries partagent environ 80% d'homologie au niveau de leur ADN génomique (148). L'acquisition d'un premier système de sécrétion de type 3 (SST3-1), survenue il y a environ 25 à 40 millions d'années par un ancêtre commun, a joué un rôle-clé dans l'évolution des salmonelles (14). En effet, cet élément génétique a permis la colonisation intestinale des vertébrés à sang froid par *Salmonella,* ainsi que de développer sa capacité d'être une bactérie pathogène intracellulaire facultative (12). Le second évènement d'importance a été l'acquisition d'un deuxième système de sécrétion de type 3 (SST3-2) permettant la survie à l'intérieur des macrophages et offrant la capacité de dissémination systémique (12, 175). Cet ilôt de pathogénicité se retrouve uniquement chez l'espèce *S. enterica* et permet d'expliquer en partie la diversification du genre *Salmonella* en 2 espèces distinctes (130, 207). Ainsi, l'absence de cet ilôt chez *S. bongori* restreint sa colonisation aux intestins des animaux à sang froid (89, 207). Le 3^{e} évènement évolutif a été l'obtention de la capacité d'effectuer la variation de phase entre 2 sous-unités structurales du flagelle, FliC et FljB (199). Puisque les flagelles sont facilement reconnus par le système immunitaire de l'hôte, cette variation antigénique permet à la bactérie d'être plus efficace pour s'évader de cette reconnaissance immunologique. Finalement, la capacité de colonisation des animaux à sang chaud retrouvée chez la sous-espèce I de *S. enterica* constitue une étape majeure dans son évolution qui lui a permis d'infecter une panoplie d'espèces. La colonisation des mammifères et des oiseaux ne peut s'expliquer par l'acquisition d'un seul déterminant génétique (14). Les vertébrés à sang froid possèdent un

système immunitaire moins bien développé que les mammifères. La pathogénicité des sérovars de la sous-espèce I provient nécessairement de leur résistance accrue face aux défenses de leurs hôtes, notamment la présence de macrophages dans les ganglions lymphatiques mésentériques (14). Plus de 99% des cas de maladies humaines sont associés à la sous-espèce I de *S. enterica* (21).

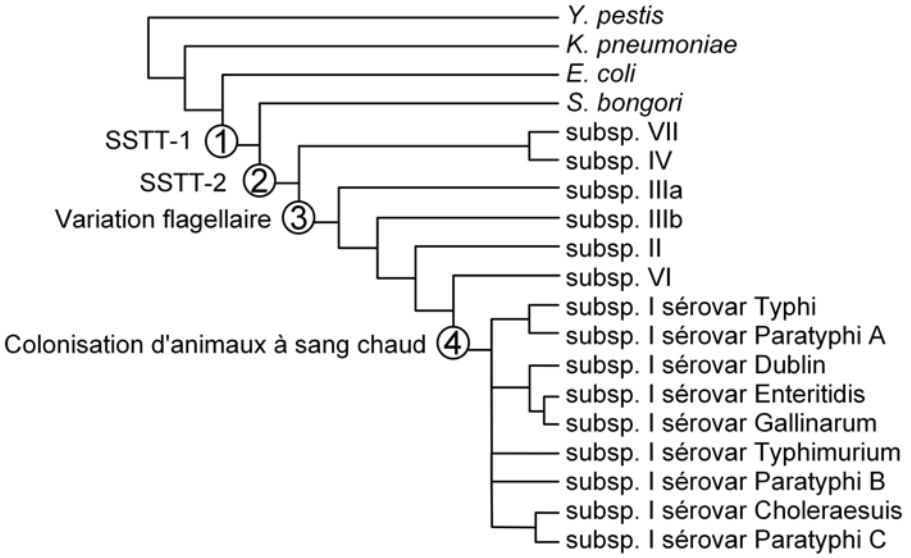

Figure 1.1. Arbre phylogénétique de *Salmonella*. Les 4 étapes majeures qui ont mené à la diversification de *Salmonella* des autres entérobactéries sont identifiées. 1) Acquisition du premier système de sécrétion de type 3 situé sur le SPI-1 et requis pour l'invasion cellulaire. 2) Acquisition du deuxième système de sécrétion de type 3 utilisé pour la survie à l'intérieur des macrophages. 3) Obtention de la capacité d'effectuer la variation flagellaire entre les sous-unités structurales FliC et FljB du flagelle. 4) Développement de la capacité de colonisation des animaux à sang chaud. Subsp. signifie sous-espèce et font toutes partie de l'espèce *Salmonella enterica*. Figure conçue à partir de (14, 73, 200).

1.1.2.1. Spectres d'hôtes observés chez *S. enterica* subsp. *enterica*

Une caractéristique intéressante des sérovars de *S. enterica* subsp. *enterica* est l'impressionnante diversité observée au niveau de leurs spectres d'hôtes. Certains sérovars surnommés les « généralistes » possèdent la capacité de coloniser ou d'infecter un large spectre d'hôte, allant des mammifères aux animaux à sang froid (voir Tableau II). D'autres

sérovars s'associent de préférence à un seul hôte, mais sont tout de même capables d'infecter d'autres espèces et sont donc considérés comme étant « adaptés ». Finalement, certains sérovars infectent exclusivement un seul hôte sont classifiés comme étant « hôte-spécifique ». Les hypothèses pouvant expliquer cette variation dans le spectre d'hôte seront discutées dans la section 1.1.3.

Tableau II. Spectres d'hôtes observés pour les sérovars les plus communs de *S. enterica* subsp. *enterica* (62, 191, 280).

Classification	Sérovars	Hôtes naturels	Hôtes occasionnels
Généralistes	Typhimurium	Humains, volaille, porcins, rongeurs, bovins	Reptiles
	Enteritidis	Humains, volaille, rongeurs	Porcins, bovins, reptiles
Adaptés à un hôte	Choleraesuis	Porcins	Humains
	Dublin	Bovins	Humains et ovins
	Paratyphi C	Humains	Souris
Spécifiques à un hôte	Typhi	Humains	Aucun
	Paratyphi A	Humains	Aucun
	Gallinarum	Volaille	Aucun
	Typhisuis	Porcins	Aucun

1.1.3. Comparaison génétique de sérovars de *S. enterica*

Le séquençage génomique des sérovars de *Salmonella enterica* a fourni de précieuses informations pouvant expliquer les différences observées dans leurs profils de colonisation d'hôtes. Il semble que l'effet combiné de l'acquisition de gènes par transfert horizontal et la perte de fonction de plusieurs gènes puissent être responsables de la divergence observée dans l'évolution de ces bactéries (99, 213).

Le transfert horizontal de gènes peut s'effectuer par au moins trois mécanismes bien définis : conjugaison, transformation, transduction. La conjugaison s'effectue lorsqu'il y a un contact direct entre une cellule donneuse et une receveuse et le transfert d'ADN se fait généralement par un plasmide ou un transposon conjugatif (199). Lorsque l'ADN est recueilli directement de l'environnement par la cellule receveuse, il s'agit de transformation

(233). La transduction implique le transfert d'ADN par un phage ayant incorporé de l'ADN bactérien lors de l'encapsidation de son génome viral (233). Lorsque le phage s'intègre dans le génome de la cellule receveuse, il y a formation d'un prophage dans un processus appelé conversion lysogénique (199). Dans les dernières années, il a été démontré que la formation de vésicules membranaires par *E. coli* O157:H7 permet aussi le transfert de gènes de virulence et de résistance aux antibiotiques à d'autres bactéries, notamment *Salmonella* (269).

1.1.3.1. Ilôts de pathogénicité

Chez *Salmonella*, la majorité des facteurs de virulence sont retrouvés dans des régions génomiques appelées « *Salmonella* pathogenicity islands » (SPIs) qui peuvent être distinguées par plusieurs caractéristiques communes. Tout d'abord, les sites préférés d'intégration de ces éléments génétiques sont souvent situés entre deux ARNt : les séquences des ARNt sont fortement conservées entre les différentes espèces et elles s'y retrouvent en plusieurs copies (84). Des séquences d'éléments impliqués dans la mobilité de l'ADN, comme les intégrases, les transposases, les répétitions directes et des gènes de bactériophages sont généralement retrouvées dans les SPIs (199). Finalement, le contenu en G + C des SPIs diffère grandement du restant du génome de *Salmonella* et ces insertions sont habituellement absentes du génome d'*E. coli* (199). L'intégration dans le génome de la bactérie élimine la nécessité d'une origine de réplication autonome requise pour les plasmides et permet la stabilité (84). L'acquisition de ces différents éléments génétiques pourrait toutefois être nocive pour la viabilité de *Salmonella* en absence de régulation serrée. Une souche de *Salmonella* possédant une délétion dans le gène *hns* n'est pas viable, à moins d'être accompagnée de délétions dans les gènes régulateurs *rpoS* et *phoP* (162). Il a été démontré que la protéine H-NS (*Histone-like Nucleoid Structuring protein*) réprime la transcription des séquences d'ADN de source exogène récemment acquises en liant les séquences renfermant un contenu en G + C faible (162). Par exemple, l'expression du SPI-2 chez *Salmonella* provoque un ralentissement de croissance pouvant être détrimental lors

de l'infection; H-NS réprime cet ilôt tant qu'il n'est pas requis et permet un taux de croissance optimal (140).

L'acquisition de gènes par transfert horizontal a certainement eu une contribution considérable dans l'évolution des sérovars de *Salmonella*. En effet, les sérovars Typhi et Typhimurium partagent plus de 90% d'homologie génomique (148), mais causent des pathologies différentes chez l'humain (voir section 1.2). Le chromosome de *S.* Typhi contient 601 gènes qui sont absents de *S.* Typhimurium, alors que ce dernier renferme 479 gènes qui lui sont uniques (185).

Parmi ces différences, le SPI-7 de *S.* Typhi constitue la plus grosse insertion génomique avec plus de 150 gènes situés sur un total de 134 kb et pourrait expliquer son pouvoir de dissémination systémique (185). Cet ilôt renferme notamment tous les gènes nécessaires à la biosynthèse d'une capsule polysaccharidique lui permettant de pénétrer la barrière intestinale de façon silencieuse et de résister au système immunitaire, ainsi qu'un pilus de type IV utilisé pour l'invasion cellulaire (185, 195, 276). En général, *S.* Dublin infecte les bovins, mais certaines souches ont acquis récemment par transfert horizontal un ilôt similaire au SPI-7 de *S.* Typhi lui permettant de causer une fièvre typhoïde chez l'humain (156, 195, 227). Cependant, la présence du SPI-7, en particulier la capsule Vi, ne peut expliquer à elle seule la différence de pathologies associées à *S.* Typhi et *S.* Typhimurium. En effet, certaines souches virulentes de *S.* Typhi isolées de patients ne possèdent pas la capsule ou le SPI-7 (7, 146, 160). De plus, il a été démontré qu'une souche possédant une délétion dans le locus responsable de la synthèse de la capsule, ainsi que dans le gène *galE*, provoque tout de même le développement de la fièvre typhoïde chez l'humain (101). *S.* Paratyphi A et B peuvent aussi provoquer une fièvre typhoïde chez l'humain, mais ne possèdent pas les gènes de biosynthèse de la capsule Vi (29).

Deux toxines sont retrouvées uniquement chez le sérovar *S.* Typhi. La toxine CdtB située sur le SPI-11 induit un arrêt du cycle cellulaire de la cellule de l'hôte en infligeant des dommages à l'ADN (86). La toxine ClyA située sur le SPI-18, aussi connue sous les noms HlyE et SheA, forme des pores dans les membranes cellulaires, contribue à la

cytotoxicité de *S*. Typhi envers les cellules épithéliales et semble contrôler la réplication dans les macrophages (66). Ces deux toxines sont contrôlées positivement par le régulateur à deux composantes PhoP/PhoQ (28).

De son côté, *S*. Typhimurium possède le plasmide de virulence pSLT transmissible d'environ 90 kb qui est nécessaire pour la virulence dans la souris et absent de *S*. Typhi (3, 111). Ce plasmide est souvent retrouvé chez les isolats humains et code notamment pour la toxine SpvB qui inhibe la polymérisation de l'actine, ainsi que le fimbria Pef requis pour l'adhésion intestinale chez la souris (13, 24, 74). Cependant, l'insertion de ce plasmide chez *S*. Typhi n'est pas suffisante pour lui permettre d'infecter la souris (217). Pour obtenir plus d'informations concernant les différences génétiques au niveau des SPIs de *S*. Typhi et *S*. Typhimurium, consulter l'annexe I de Sabbagh *et al*. 2010.

1.1.3.2. Pseudogènes

Le séquençage des différents sérovars de *Salmonella* a révélé une caractéristique commune aux sérovars possédant un spectre d'hôte restreint : il existe une forte tendance vers la dégradation génomique, c'est-à-dire l'accumulation d'inactivations génétiques. Les évènements menant à une insertion, une délétion ou une mutation nonsense prématurée dans un cadre de lecture ouvert induisent généralement la perte de fonction de ces gènes qui sont donc considérés comme des pseudogènes. Le génome des organismes intracellulaires obligatoires ou ayant un spectre d'hôte restreint contient en moyenne davantage de pseudogènes que les organismes ubiquitaires (4, 37, 147, 186, 248). Cette observation appuie l'hypothèse que les gènes non-utilisés finissent par accumuler des mutations inactivatrices. Il existe effectivement une très forte relation entre le nombre de pseudogènes retrouvés dans un génome et le spectre d'hôtes des sérovars de *Salmonella* (Tableau III).

L'adaptation à une nouvelle niche plus restreinte explique possiblement l'inutilité de certains gènes et leur inactivation. D'un autre côté, il est aussi possible que l'apparition d'une mutation inactivatrice empêche une bactérie pathogène de pouvoir coloniser plusieurs hôtes. En regardant la classification des pseudogènes retrouvés chez les quatre sérovars au spectre d'hôte unique, près du tiers des gènes inactivés font partie des protéines

de membrane/surface, suivis par les régulateurs et les gènes associés à la pathogénicité/adaptation/chaperonne (98). Ces données suggèrent que la pression évolutive exercée par le système immunitaire de leur hôte respectif pourrait en partie être responsable de cette dégénérescence (100). Par exemple, plusieurs facteurs de virulence associés à la colonisation intestinale prolongée chez les animaux (*ratB*, *sivH*, *shdA*) (120) sont devenus pseudogènes chez les sérovars à spectre d'hôte unique (147). En effet, l'association à long terme avec les intestins de leur hôte n'est pas requise pour les sérovars qui produisent une maladie systémique (100).

Les sérovars Typhi et Paratyphi A, infectant les humains seulement, partagent 66 pseudogènes communs (100). Ces deux sérovars proviennent d'un ancêtre commun (voir Fig. 1.1), mais l'inactivation commune de certains gènes s'est réalisée de façon indépendante (100). Un exemple intéressant parmi les sérovars de *Salmonella* est la divergence évolutive de *S.* Paratyphi C de son ancêtre *S.* Choleraesuis (Fig. 1.1) et sa convergence avec *S.* Typhi en ce qui a trait à sa pathogenèse systémique chez l'humain (Tableau II). La comparaison génomique de plusieurs sérovars n'a pas permis d'identifier les facteurs de virulence responsables de la fièvre typhoïde chez l'humain (98, 138). Néanmoins, ces études ont permis de confirmer l'évolution génomique constante des différents sérovars de *Salmonella* lors de l'adaptation à une niche, que ce soit par l'acquisition ou l'inactivation de leur matériel génétique.

Tableau III. Relation entre le nombre de pseudogènes retrouvés dans un génome et le spectre d'hôtes de différents sérovars de *Salmonella*

Sérovars	Spectre d'hôtes	Pseudogènes	Taille du génome	Référence
Typhimurium	Généraliste	39	4,86 Mb	(148)
Enteritidis	Généraliste	113	4,69 Mb	(247)
Paratyphi C	Adapté	149	4,83 Mb	(138)
Choleraesuis	Adapté	151	4,76 Mb	(34)
Paratyphi A	Unique	173	4,59 Mb	(147)
Typhisuis	Unique	190	4,65 Mb	(98)
Typhi	Unique	204	4,81 Mb	(185)
Gallinarum	Unique	309	4,66 Mb	(247)

1.2. Salmonelloses

Les infections causées par les différents sérovars de *Salmonella* peuvent être considérées comme étant localisées, systémiques ou asymptomatiques. Les salmonelloses s'acquièrent principalement suite à l'ingestion d'eau ou d'aliments contaminés, mais le contact direct avec des animaux contaminés constitue une autre voie d'acquisition de la bactérie.

1.2.1. Infections localisées

1.2.1.1. Pathogenèse de *S.* Typhimurium chez l'humain

L'ingestion de *S.* Typhimurium par une voie de contamination fécale-orale mène généralement au développement d'une gastro-entérite chez l'humain. Avant d'atteindre les cellules intestinales, la bactérie doit d'abord affronter divers environnements hostiles, dont le premier est de survivre au pH fortement acide de l'estomac. L'exposition de la bactérie à un pH modéré situé entre 5.5 et 6.0 mène à l'induction d'une réponse de tolérance à l'acide principalement médiée par la protéine Fur, le régulateur global du fer, ainsi que le facteur sigma alternatif *rpoS* et le régulateur à deux-composantes PhoP/PhoQ (5, 72, 78). Suite à son entrée dans le petit intestin, *Salmonella* doit d'abord réussir à traverser l'épaisse couche de mucus et empêcher son élimination par le péristaltisme intestinal. *Salmonella* possède 6 à 8 flagelles péritriches lui permettant d'être mobile et d'atteindre la surface apicale des cellules intestinales (92). Dans la lumière intestinale, la bactérie doit faire face à divers agents bactéricides du système immunitaire inné tels les enzymes digestives, les sels biliaires, les anticorps IgA sécrétoires et les peptides antimicrobiens, en plus de devoir compétitionner avec la flore intestinale de son hôte (154, 202, 218, 228).

L'attachement spécifique aux cellules de l'hôte constitue une étape cruciale pour établir une infection productive. Plusieurs structures de surfaces fimbriaires et afimbriaires permettent d'établir un contact rapproché avec les cellules de l'hôte ou les composants de la matrice extracellulaire afin de le coloniser (voir section 1.4). Trois modes d'invasion des

cellules intestinales peuvent être utilisés par *Salmonella* pour atteindre l'épithélium sous-jacent (Fig. 1.2). Le mode d'entrée privilégié se fait par l'interaction avec les cellules M situées dans les plaques de Peyer (110). Ces cellules ont comme rôle d'échantillonner les antigènes intestinaux par pinocytose pour ensuite les présenter aux cellules lymphoïdes situées sous les plaques de Peyer. Ce processus est essentiel dans le développement d'une immunité mucosale efficace. Le deuxième moyen d'entrée peut survenir suite à l'adhésion de la bactérie à la surface des entérocytes. *Salmonella* peut forcer son invasion par l'utilisation de son SST3-1 qui injecte des effecteurs protéiques directement dans le cytoplasme des cellules. Cet évènement perturbe les microvilli des entérocytes, provoque un remaniement de l'actine de l'hôte qui génère une ondulation de la membrane et englobe les bactéries adhérées dans des vésicules. Les bactéries peuvent aussi traverser la barrière épithéliale de façon passive en étant capturées par les cellules dendritiques (209). Celles-ci sont situées du côté baso-latéral et peuvent produire des pseudopodes entre les entérocytes pour aller chercher directement les bactéries retrouvées sur le côté apical. Lorsque *Salmonella* se retrouve dans un environnement intracellulaire (cellules épithéliales et monocytes), l'utilisation de ses deux SST3 lui permet de modifier son phagosome en SCV (*Salmonella* containing vacuole) pour empêcher sa destruction et promouvoir sa croissance (142).

Chez l'humain, *S.* Typhimurium produit une gastro-entérite caractérisée par une forte réaction inflammatoire intestinale et le recrutement de neutrophiles au site de l'infection (91). La reconnaissance de la flagelline par le TLR-5 (*toll-like receptor* 5) situé du côté baso-latéral des entérocytes est responsable de leur relâche d'interleukine 8 (IL), puis d'IL-1β par les macrophages provoquant l'inflammation (81, 153). Cette réaction provoque de l'œdème, de la nécrose de la muqueuse intestinale, de la sécrétion d'ions chlorure et de l'accumulation de fluides menant à la diarrhée (275). L'infection par *S.* Typhimurium chez l'humain se limite généralement aux intestins et aux ganglions mésentériques grâce à la réponse immunitaire rapide et à sa survie amoindrie dans les macrophages humains (204, 224). Cependant, les patients possédant un système

immunitaire affaibli, comme ceux affectés par le VIH, peuvent tout de même développer une infection systémique par *S.* Typhimurium (204).

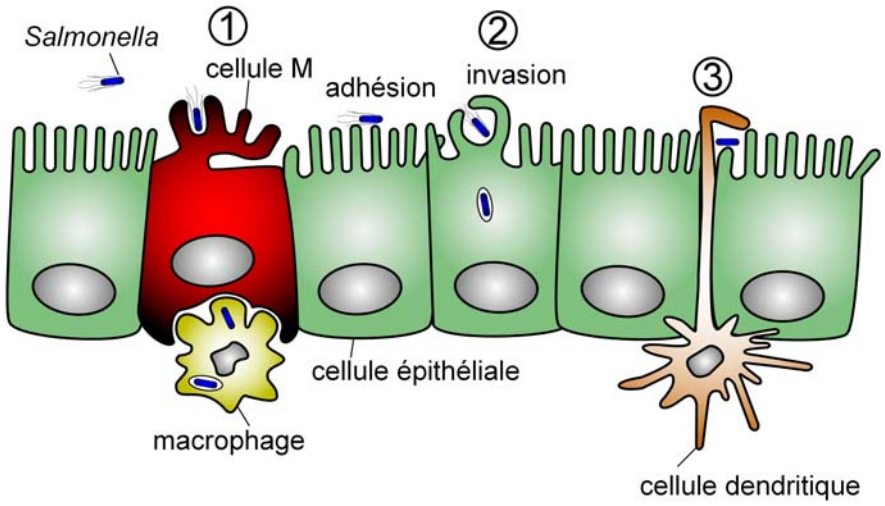

Figure 1.2. Interactions et mécanismes d'invasion utilisés par *Salmonella* pour franchir la barrière intestinale. 1) *Salmonella* transite par pinocytose à travers les cellules M et est phagocyté par les macrophages de l'épithélium sous-jacent. 2) Suite à son adhésion, *Salmonella* force son entrée dans les entérocytes en injectant des effecteurs du SSTT-1 dans le cytoplasme de la cellule provoquant le remaniement de l'actine. 3) Les cellules dendritiques peuvent capturer les bactéries dans la lumière intestinale par l'extension de pseudopodes. Figure inspirée de (216).

1.2.1.2. Prévalence, traitement et prévention

Les gastro-entérites causées par *Salmonella* constituent un problème de santé publique important à la fois dans les pays développés et en voie de développement avec une estimation de 94 millions de cas annuels, dont 155 000 décès (141). Ce nombre d'infections peut être fortement sous-estimé par le fait qu'une gastro-entérite justifie rarement le besoin d'aller à l'hôpital pour recevoir un traitement. Les symptômes de maux de ventre, vomissements, diarrhée ou fièvre se déclarent de 6 à 72 heures suivant l'ingestion de la bactérie et peuvent durer jusqu'à sept jours (95). Dans le cas d'individus sains infectés, il

ne devrait pas y avoir de traitement antibiotique, puisque celui-ci peut prolonger la durée d'excrétion bactérienne dans les fèces (163). Les meilleurs moyens de prévention des gastro-entérites à *Salmonella* sont une bonne manipulation de la nourriture contaminée, l'éducation populaire et tenter de contrôler les animaux porteurs. En effet, le réservoir principal de *S.* Typhimurium est retrouvé chez les animaux de la ferme utilisés dans l'industrie alimentaire (119). Ainsi, l'élimination des animaux porteurs par le biais de leur vaccination constitue un moyen de prévention déjà disponible pour immuniser la volaille contre les sérovars Typhimurium et Enteritidis au Canada (1).

1.2.2. Infections systémiques

1.2.2.1. Pathogenèse de *S.* Typhi chez l'humain

Les étapes initiales reliées à la pathogenèse de *S.* Typhi chez l'humain sont comparables à celles retrouvées pour *S.* Typhimurium. Les premières différences notables se produisent au niveau de l'interaction avec la barrière intestinale. Contrairement à *S.* Typhimurium, *S.* Typhi possède une capsule l'empêchant d'être reconnu par le système immunitaire humain via les TLR-4 et 5 activés par le LPS et la flagelline respectivement (264, 265). La production de la capsule est contrôlée de façon positive par le régulateur TviA suite à l'entrée de la bactérie dans les cellules intestinales (254). TviA régule aussi négativement la production de flagelles (265). Ainsi, l'infection par *S.* Typhi ne mène pas à la sécrétion d'IL-8 et n'induit pas d'inflammation intestinale. Cette caractéristique explique en partie la dissémination systémique de *S.* Typhi chez l'humain. L'absence ou la faible reconnaissance immunitaire au niveau de la barrière intestinale lui permet de la franchir et d'infecter les macrophages sous-jacents. Puisque *S.* Typhi survit dans les macrophages humains, la bactérie est véhiculée vers les ganglions lymphatiques, le foie et la rate de son hôte où elle peut s'y multiplier (257). Après une période d'incubation de 7 à 14 jours, les bactéries sont relâchées dans le sang puis une infection secondaire se produit dans le foie, la rate, la moelle osseuse, la vésicule biliaire et les plaques de Peyer (187). C'est à ce moment que les symptômes de fièvre prolongée, maux de tête et de ventre, ainsi que de la léthargie

sont ressentis (102). La colonisation de la vésicule biliaire produit la relâche de bactéries dans les selles, mais peut aussi mener à une seconde invasion de l'intestin. Les complications médicales les plus communes observées suite à cette infection sont des saignements intestinaux, des perforations intestinales et des encéphalopathies pouvant mener jusqu'à la mort (102).

1.2.2.2. Prévalence, traitement et prévention

Les infections causées par *S.* Typhi se produisent principalement dans les pays en voie de développement où les conditions sanitaires et l'accès à l'eau potable sont déficients (Fig.1.4). Il y a environ 22 millions de cas par année dont plus de 200 000 décès (42). Cependant, la véritable ampleur des cas d'infections peut être grandement sous-estimée en raison du manque de déclaration obligatoire ou d'outils de détection efficaces (42). Sans traitement, la mortalité atteint 5 à 30% de personnes infectées (102). Ainsi, la thérapie par antibiotique devient indispensable pour sauver la vie du patient. Le meilleur moyen de prévention disponible contre la fièvre typhoïde réside dans l'amélioration des conditions sanitaires, mais la vaccination peut aussi être un bon moyen de contrôler les épidémies (189, 267). Le premier vaccin utilisé consistait de bactéries inactivées par l'acétone, l'alcool ou la chaleur et a été introduit en 1896 (85). Celui-ci était très efficace, mais l'apparition d'effets secondaires indésirables tels de la fièvre, des maux de tête et des réactions cutanées, a forcé la création de nouveaux vaccins (189). En ce moment, l'injection de la capsule Vi ou la prise orale de la souche vivante atténuée Ty21a sont les deux vaccins utilisés contre *S.* Typhi (Tableau IV). Leur efficacité varie de 50 à 94%, mais aucun de ces vaccins n'offre de protection à long terme (189). Plusieurs autres vaccins sont présentement en essais dans le but d'améliorer leur efficacité et protection à long terme (189). Les caractéristiques d'un vaccin idéal seraient de pouvoir être conservé à température ambiante, être donné de façon orale en une seule dose et offrir une protection à long terme même chez les jeunes enfants.

Tableau IV. Comparaison des vaccins utilisés pour prévenir la fièvre typhoïde (189).

Vaccin	Description	Avantages	Inconvénients
Bactéries entières tuées	Bactéries entières tuées à l'acétone, alcool ou chaleur	Sans danger pour patients immunosupprimés Conservation à température ambiante	Plusieurs réactions indésirables Injectable
Ty21a	Vaccin vivant atténué	Administration orale Peu de réactions secondaires	Plusieurs doses nécessaires Capsule Vi non exprimée Majorité des mutations inconnues Conservation à 4°C
Polysaccharide Vi	Capsule Vi purifiée	Sans danger pour patients immunosupprimés	Injectable Conservation à 4°C Pas efficace chez les jeunes enfants

1.2.3. Infections asymptomatiques

La colonisation intestinale par *Salmonella* ne mène pas toujours au développement d'infections localisées ou systémiques. Plusieurs animaux destinés à la consommation humaine peuvent être colonisés de façon asymptomatique par *Salmonella* et constituer un réservoir majeur de propagation de la bactérie. Le seul réservoir de contamination par *S.* Typhi identifié à ce jour provient des porteurs asymptomatiques humains. Suite à l'infection, de 1 à 5% des patients deviennent porteurs chroniques de la bactérie dû à la formation de biofilms associés à la vésicule biliaire (40, 131, 150). Ces personnes peuvent excréter activement *S.* Typhi dans leur urine et leurs fèces, parfois durant des dizaines d'années (17, 232). Les biofilms sont des structures complexes qui protègent les bactéries des stress environnementaux, des désinfectants, du système immunitaire et des antibiotiques en formant une barrière physique et en induisant l'état de dormance (25, 96). Chez *Salmonella*, les biofilms augmentent la résistance à la ciprofloxacine en empêchant sa diffusion et peuvent laisser des cellules persistantes qui reforment un nouveau biofilm suite

au retrait de l'antibiotique (206, 242). Récemment, la présence de calculs biliaires a été reconnue comme étant un facteur majeur prédisposant à la formation de biofilms (40). Les porteurs constituent une menace constante pour la santé publique et leur traitement contribuerait largement à la diminution du nombre de cas d'infections. Cependant, environ 25% des porteurs asymptomatiques ne ressentent aucun symptôme lors de la phase aiguë de l'infection, ce qui complique grandement leur identification (187). De plus, l'utilisation d'antibiotiques permet rarement de guérir les porteurs asymptomatiques obligeant le retrait des calculs biliaires, voire l'enlèvement complet de la vésicule biliaire (238). Cette intervention est risquée pour la vie du patient, onéreuse et élimine *S.* Typhi chez seulement deux tiers des patients (52). Chez certains porteurs chroniques, l'excrétion de *S.* Typhi est observée malgré l'enlèvement de la vésicule biliaire, suggérant qu'il existe d'autres foyers d'infection, comme le foie et les ganglions lymphatiques mésentériques (83, 161).

1.2.4. Résistance aux antibiotiques chez *S.* Typhi

1.2.4.1. Historique

Dès la première utilisation d'antibiotiques contre *S.* Typhi en 1948, une course contre la montre s'est installée entre leur efficacité de traitement et l'apparition de résistances (Fig. 1.3). Les souches multi-résistantes aux antibiotiques (MDR) de première ligne, tels l'ampicilline, le chloramphénicol et le cotrimoxazole (triméthoprime-sulfaméthoxazole) ont été répertoriées en 1972 au Mexique et en Inde, puis sont devenues un problème grave vers la fin des années 1980 (178). La prévalence mondiale des souches MDR a forcé l'utilisation de nouveaux agents antimicrobiens, soit les fluoroquinolones, telles la ciprofloxacine et l'acide nalidixique, ainsi que les céphalosporines de 3^{e} génération pour les enfants (260, 263) (Fig. 1.4). Ces antibiotiques sont actuellement recommandés pour le traitement de la fièvre typhoïde puisqu'ils sont efficaces, bien tolérés, peu coûteux et peuvent être donnés de façon orale ou intraveineuse (267). Cependant, l'apparition de souches fortement résistantes aux fluoroquinolones en Asie a été confirmée en 1997 et constitue une donnée inquiétante (258). Par exemple, plus de 88% des isolats au Vietnam

sont considérés MDR et 93% sont résistants à l'acide nalidixique (188). L'azithromycine peut être utilisée comme drogue alternative dans les cas de multi-résistance, mais des isolats résistants ont déjà été répertoriés (26, 271).

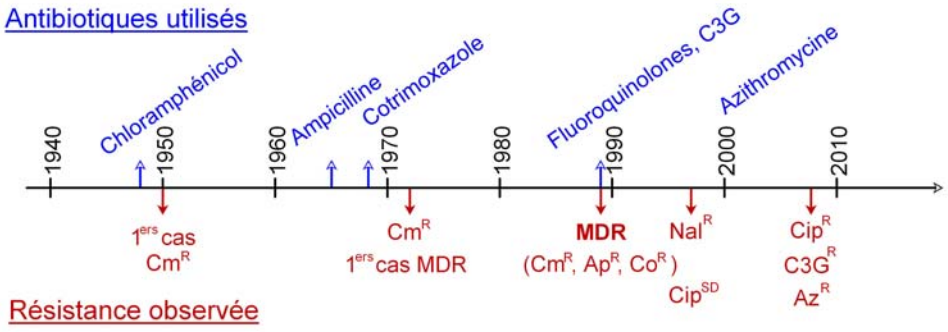

Figure 1.3. Antibiotiques utilisés pour traiter la fièvre typhoïde et apparition de résistance chez les isolats de *S.* Typhi (263, 271). Cm (chloramphénicol), MDR (*multi-drug resistant* : Cm^R, Ap^R, Co^R), Ap (Ampicilline), Nal (acide nalidixique), Cip (Ciprofloxacine), C3G (céphalosporines de 3^e génération), Co (cotrimoxazole), Az (azithromycine), R (résistant), SD (sensibilité diminuée).

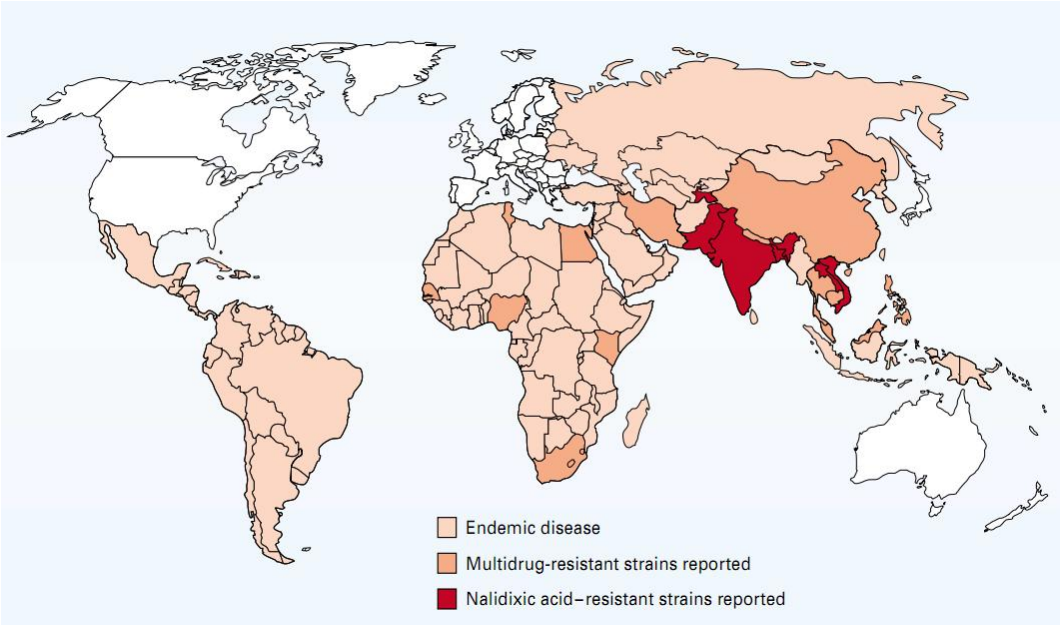

Figure 1.4. Zones endémiques de la fièvre typhoïde causée par *S.* Typhi. En plus de montrer les pays touchés par la maladie, la carte montre les pays où des souches MDR ou résistantes à l'acide nalidixique ont été répertoriées de 1990 à 2002. Figure provenant de (187) et reproduite avec la permission du *New England Journal of Medecine*.

1.2.4.2. Mécanismes de résistance

La résistance aux antibiotiques chez *S.* Typhi peut être due à l'acquisition d'un plasmide de résistance ou à une mutation génomique. Les souches possédant le plasmide pHCM1 transférable et stable de 218 kb deviennent multi-résistantes à l'ampicilline, au chloramphénicol, à la tétracycline, à la streptomycine et aux sulphonamides (185, 259). La formation de pHCM1 a été engendrée par plusieurs évènements séquentiels d'insertion génétique réalisés par les intégrases et les transposases retrouvées sur ce plasmide (185). Parmi les résistances associées aux souches MDR de *S.* Typhi, l'ampicilline qui cible la transpeptidation du peptidoglycan est inactivée par le produit du gène *bla* codant pour une β-lactamase (215). L'inactivation du chloramphénicol, qui inhibe la traduction en se liant à la sous-unité 50S du ribosome, est réalisée par une chloramphénicol-acétyltransférase (*cat*I). Le cotrimoxazole cible la dihydroptéroate synthase impliquée dans la synthèse de

l'ADN et le gène *sul*II du plasmide pHCM1 code pour une variante résistante de cet enzyme (87).

Pour ce qui est des fluoroquinolones, une seule mutation dans le gène *gyrA* codant pour la sous-unité A de la gyrase confère la résistance à l'acide nalidixique et induit une diminution de la sensibilité à la ciprofloxacine (211, 263). L'accumulation d'une 2^{e} mutation dans *gyrA* suivie d'une autre mutation dans le gène *parC* codant pour une sous-unité de la topoisomérase IV confère à cette souche une forte résistance à la ciprofloxacine (51). Récemment, la présence de plasmides *qnr* médiant la production de protéines qui protègent l'ADN gyrase et la topoisomérase IV, en plus de produire des pompes à efflux sortant la ciprofloxacine de la bactérie, a été répertoriée (27, 116). L'apparition récente de la souche multi-résistante NDM-1 (*New Delhi Multiresistant*) d'*Escherichia coli* dans les zones endémiques de la fièvre typhoïde représente un danger potentiel pour la santé publique. En effet, lorsque les résistances sont retrouvées sur un plasmide, il y a une forte possibilité de transfert entre *E. coli* et *Salmonella*.

1.3. Modèles d'étude pour identifier les facteurs de virulence impliqués dans la fièvre typhoïde

1.3.1. *S.* Typhimurium chez la souris

Puisque *S.* Typhi est spécifique à l'humain, il n'existe pas de modèle animal représentatif dans la nature (Tableau V). Les données concernant l'établissement de la fièvre typhoïde proviennent principalement de l'infection de *S.* Typhimurium chez la souris. Certaines lignées murines (C57BL/6 et BALB/c) développent une fièvre entérique mortelle, avec des symptômes semblables à ce qui est retrouvé chez l'humain, suite à l'infection par *S.* Typhimurium. Leur susceptibilité provient d'une mutation dans le gène *Nramp1* (*Slc11a1*) qui code pour un transporteur de métaux divalents localisé dans les phagosomes des macrophages et qui limite l'accès des micro-nutriments essentiels aux bactéries phagocytées (18). Les lignées de souris possédant l'allèle sauvage de *Nramp1*

permettent d'étudier l'infection chronique des tissus, semblable à ce qui survient chez les porteurs asymptomatiques de *S.* Typhi (155). De plus, la destruction de la flore normale intestinale de la souris par un traitement à la streptomycine permet de recréer la gastro-entérite humaine (11).

De nombreux avantages justifient l'utilisation du modèle murin de fièvre entérique, notamment l'accessibilité, les faibles coûts de l'utilisation d'un modèle de souris, la reproduction de la maladie observée chez l'humain et la présence de tous les types cellulaires (par exemple : cellules épithéliales et du système immunitaire). La génétique d'une souris peut être modifiée facilement pour découvrir les facteurs de l'hôte impliqués dans la susceptibilité à *Salmonella* (57). Cet outil a été fort utile pour découvrir les facteurs de virulence cruciaux pour établir une infection systémique par *S.* Typhimurium, mais possède néanmoins certaines limitations. Le principal inconvénient relié à ces études est qu'il ne permet pas d'étudier les facteurs de virulence qui sont retrouvés uniquement dans le génome de *S.* Typhi. De plus, la génétique d'une souris diffère considérablement de celle de l'humain; certaines souches avirulentes chez la souris ne le sont pas une fois testées chez l'humain (50, 101, 236). Par exemple, aucune association allélique n'a été observée entre le gène *Nramp1* et la susceptibilité à la fièvre typhoïde chez l'humain (60). L'introduction des gènes uniques à *S.* Typhi dans le sérovar Typhimurium constitue une autre approche pour étudier les facteurs de virulence spécifiques à *S.* Typhi. Par exemple, cette méthode a permis de découvrir le profil d'expression et l'importance de la capsule Vi, ainsi que son régulateur TviA, lors de l'établissement d'une infection systémique (voir section 1.4.4) (88, 205, 254, 265).

1.3.2. Souris humanisées

Récemment, plusieurs laboratoires ont développé un modèle de souris humanisées (134, 152, 236). Leurs réussites sont basées sur l'introduction de cellules du système immunitaire humain dans des souris immunodéficientes irradiées. Les cellules hématopoïétiques proviennent soit de sang de cordons ombilicaux (134, 152) ou de foies

fœtaux (236). Ce modèle se rapproche davantage de la réalité puisqu'il permet d'utiliser directement *S*. Typhi dans la souris. Cependant, l'injection doit se faire directement dans la cavité intrapéritonéale et ne permet pas d'étudier les premières étapes de l'infection, notamment l'interaction avec la barrière intestinale. Pour le moment, ces modèles sont peu abordables, requièrent des manipulations complexes et provoquent une mortalité rapide chez les souris (134, 255). Les données obtenues de ces modèles constituent toutefois une avancée majeure vers la compréhension de la pathogenèse de *S*. Typhi dans les cellules hématopoïétiques.

1.3.3. Cellules immortalisées

Le moyen le plus simple et accessible pour les laboratoires de recherche réside dans l'utilisation de cellules immortalisées. Les lignées de cellules épithéliales et de monocytes différenciés en macrophages constituent les principaux types cellulaires impliqués dans la pathogenèse de *Salmonella* et utilisés en recherche. Tout comme les deux autres modèles mentionnés ci-haut, certains inconvénients sont reliés à cette approche.

En général, un seul type cellulaire peut être étudié à la fois, mais cette limitation peut devenir avantageuse pour identifier avec certitude la cellule-cible d'un facteur de virulence. L'épithélium intestinal est une structure en monocouche complexe caractérisée par la présence de multiples types cellulaires différenciés (entérocytes, cellules M, cellules caliciformes et de Paneth) (164). Certaines lignées de cellules épithéliales (CaCo2, T84 et MDCK) peuvent croître sur un support perméable, puis se différencier en cellules polarisées produisant des microvilli et des jonctions serrées (106). Cette différenciation peut parfois être nécessaire pour observer l'effet d'un facteur de virulence (79). Le SST3-1 utilisé par *Salmonella* pour l'invasion cellulaire a cependant été identifié grâce à la lignée de cellules non-différenciées Henle-407 (INT-407) (75). Un autre désavantage des cellules épithéliales réside dans l'absence des composants de la matrice extracellulaire, comme la mucine, qui peuvent parfois être utilisés comme molécules d'adhésion (31). De plus, la transformation de cellules saines en cellules immortalisées provoque normalement

l'introduction de plusieurs mutations génétiques pouvant amener de la variation entre les différents laboratoires de recherche.

L'utilisation de lignées de monocytes capables de se différencier en macrophages comporte moins de différences physiologiques que les cellules épithéliales. Tout d'abord, les monocytes ne requièrent pas de structures tridimensionnelles complexes pour effectuer leur différentiation cellulaire et être activés. L'utilisation d'esters de phorbol, comme le PMA, miment l'effet activateur du diacylglycérol sur la protéine kinase C et provoquent la différenciation des lignées de monocytes en macrophages (225). Ainsi, la lignée de monocytes THP-1 constitue un excellent modèle qui se rapproche des caractères observés chez les macrophages retrouvés dans les tissus humains (43, 77). De plus, il existe une très bonne corrélation observée entre les infections de lignées de monocytes différenciées et de l'hôte : les mutants considérés avirulents dans les macrophages le sont aussi chez la souris (67) et humaines (58). L'utilisation de lignées de monocytes d'origines murines et d'humains a même permis d'observer des différences majeures dans la survie des sérovars Typhi et Typhimurium dans les macrophages de leur hôte respectif (224).

Tableau V. Comparaison des modèles d'études pour la fièvre typhoïde de *Salmonella* chez l'humain (adapté de (255))

Modèle	Avantages	Inconvénients
Typhoïde murine	Infection naturelle, disponibilité de la génétique de l'hôte et des réactifs immunologiques, modèle pour la transmission fécale-orale; peu coûteux	*S.* Typhimurium ne cause pas de fièvre typhoïde chez l'humain, ne permet pas l'étude des facteurs de virulence uniques à *S.* Typhi
Souris humanisées	Permet d'infecter la souris avec *S.* Typhi, cytokines et anticorps humains produits	Requiert des manipulations complexes et variées, injection intrapéritonéale seulement, coûteux
Cellules immortalisées	Peu coûteux, manipulations simples et reproductibles, plusieurs sources disponibles	Un seul type cellulaire étudié, accumulation de mutations absente des cellules normales

1.4. Facteurs de virulence situés à la surface de *S.* Typhi

Les différentes structures retrouvées à la surface jouent un rôle crucial dans la pathogenèse bactérienne puisqu'elles permettent d'effectuer des interactions directes avec les cellules de l'hôte. Dans son génome, *S.* Typhi possède toute l'information génétique pour produire des adhésines fimbriaires et afimbriaires, une capsule, des LPS, deux systèmes de sécrétion de type 3, des flagelles ainsi qu'un système de sécrétion de type 6. Toutes ces structures possèdent des mécanismes d'expression et des rôles différents dans la virulence de *S.* Typhi qui seront détaillés dans l'article numéro 1. Cet article a été soumis en tant que chapitre de livre portant sur *Salmonella* à l'éditeur InTech. Il est présentement en processus de révision par les pairs.

Article 1 : **Forest, C. G.** and F. Daigle. 2011. Molecular armory of *S.* Typhi: deciphering the putative arsenal of our enemy. Accepté comme chapitre de livre à l'éditeur InTech.

Contribution des auteurs :

Les diverses expérimentations, les figures et le tableau, ainsi que la rédaction de l'article ont été effectuées par moi-même. France Daigle a aussi participé à l'écriture de l'article et a fourni le support financier.

CHAPITRE 1, Section 1.4. 1er article

Molecular armory of *S.* Typhi: deciphering the putative arsenal of our enemy

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1.4.1. Introduction

The outer surface of bacteria is the first to interact with host components, such as the immune system, the extracellular matrix or cells. The bacterial Gram-negative cell wall is complex and composed of an inner membrane (IM), a periplasmic space and a thin peptidoglycan layer, all surrounded by an outer membrane (OM). The OM is a bilayered structure consisting mainly of phospholipids, proteins and lipopolysaccharide (LPS) and serves as an impermeable barrier to prevent the escape of periplasmic molecules but also acts as a barrier for entry of external molecules. *Salmonella enterica* comprises more than 2500 serovars, based on three major antigens located at the cell surface : O antigen, capsule and flagella. All serovars are highly conserved genetically but have different host ranges and cause different diseases. In humans, *Salmonella* infection causes gastroenteritis, often associated with serovars Typhimurium and Enteritidis or typhoid-like disease, which is associated with serovars Typhi and Paratyphi. *S.* Typhi strains belong to serogroup D1 with the antigenic formula O:9,12; Vi+; H-d. These strains are human-restricted and besides asymptomatic carriers, no environmental reservoir is known.

S. Typhi is a monomorphic bacterium, showing very little genetic diversity (Kidgell et al., 2002) and up to 5% of its annotated coding sequences are pseudogenes (Holt et al., 2009; Parkhill et al., 2001). Genome degradation may be responsible for its host specificity;

however the *S*. Typhi genome may harbour specific genes for its systemic dispersion and survival. *S.* Typhi remains a major public health problem in developing countries. Antimicrobial resistance has become a problem in endemic regions, and it is becoming imperative to develop new vaccine strategies or discover new antimicrobial targets to combat this microorganism. Bacterial surface proteins may correspond to these targets by being immunogenic or essential for virulence. Most virulence factors are usually located within genomic locations called *Salmonella* Pathogenicity Islands (SPIs) and are tightly regulated by global regulators such as PhoP-PhoQ, RcsDBC, OmpR-EnvZ and RpoS. This review will focus on molecules localized at the outer membrane of *S.* Typhi and their role in pathogenesis. A complete analysis of adhesive molecules, such as the 12 fimbrial systems, curli, type IVB pilus, autotransporters and afimbrial adhesins will be presented. We will also discuss the importance of polysaccharides such as the Vi capsule and LPS. Furthermore, the complex surface structures generated by secretion systems, such as type three secretion systems (T3SS), flagella and T6SS that are so important for invasion, intracellular survival and to highjack the host defence system will be discussed. Finally, methods used to inhibit these adhesive structures will be described.

1.4.2. Fimbrial adhesins

Fimbriae (also called pili) are proteinaceous structures that can be observed as filaments anchored on the bacterial cell surface. These structures can mediate crucial interactions during host infection like adherence, invasion or biofilm formation, and are classified according to their mechanism of assembly. Most of the fimbriae present in *S*. Typhi genome are assembled by the chaperone/usher pathway, but there are also one representative of the nucleation/precipitation pathway (*csg*) and one type IVB pilus. This section will briefly describe each mechanism of expression and the current knowledge related to *S*. Typhi and their putative roles.

1.4.2.1. Mechanisms of fimbrial assembly

1.4.2.1.1. The chaperone/usher pathway

Twelve fimbrial systems detected in *S.* Typhi belong to the chaperone/usher pathway (CUP) assembly class (Fig. 1). A classic fimbrial operon usually harbours at least four different genes. The filaments are composed of major and minor fimbrial subunits assembled by the cooperative work of the chaperone and the usher. After translocation by the Sec general secretory pathway, the periplasmic chaperone protects the subunits and brings them to the OM usher, which specifically translocates subunits to the cell surface. Fimbrial biogenesis by the CUP pathway is a self-energized process catalyzed by both the usher and the presence of high-energy intermediates in the folding of the chaperone-subunit complexes (Jacob-Dubuisson et al., 1994; Nishiyama et al., 2008; Sauer et al., 2002; Zavialov et al., 2002). Classification based upon sequence homology between the different ushers (Nuccio & Bäumler, 2007) revealed members in the γ1- (*bcf*, *fim*, *stg*, *sth*), γ3- (*saf*, *sef*), γ4- (*sta*, *stb*, *stc*), π- (*std*, *ste*) and the α-fimbrial clades (*tcf*) in *S.* Typhi (Fig. 1).

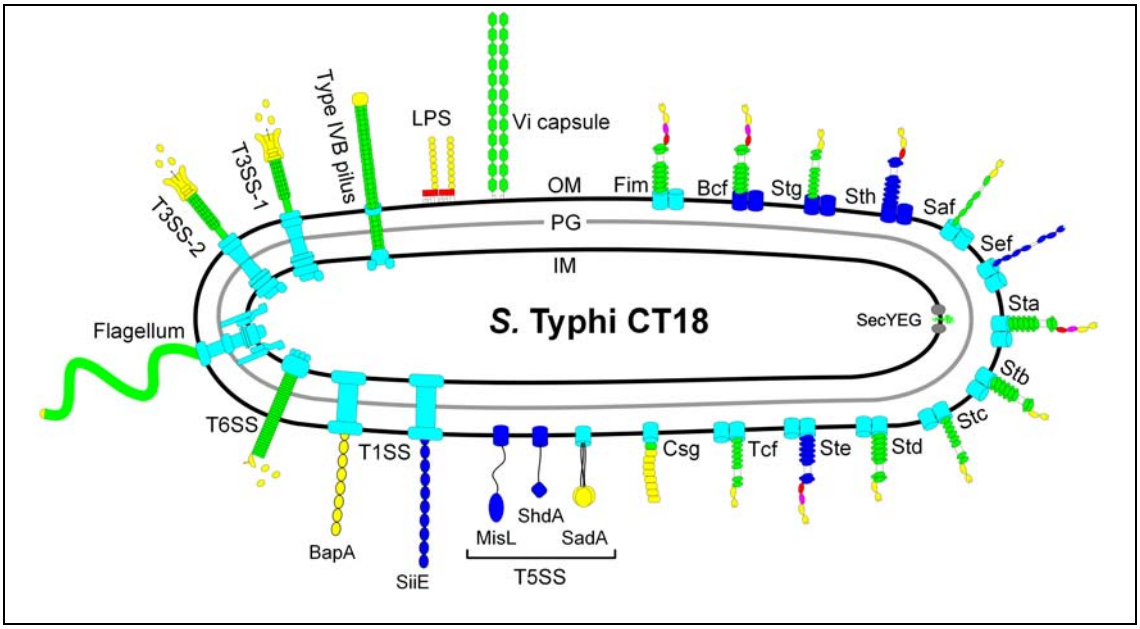

Figure 1. Schematic representation of the important surface structures identified in *S.* Typhi CT18. Adhesive components are shown in yellow, membrane components are cyan and pseudogenes are shown in blue (pseudogenes of the T6SS are not shown). The twelve representatives of the CUP are grouped in fimbrial clades and are drawn according to previous observations (Salih et al., 2008) or based on their homologues found in *E. coli* K-12 (Korea et al., 2011). STY0405 putative autotransporter, STY0351 adhesin, and PagC which are known to be implicated in virulence were omitted from the drawing, as well as STY1980 (MAM7). IM stands for inner membrane, PG for peptidoglycan and OM for outer membrane.

1.4.2.1.2. Nucleation/precipitation pathway

The thin aggregative fimbriae, also known as curli or TAFI, encoded by the *csgDEFG csgABC* gene cluster belongs to this class of adhesin. The first steps of biogenesis are similar to the CUP : after translocation by the Sec pathway, CsgA and CsgB fimbrial subunits are secreted by the CsgG outer membrane protein at the bacterial cell surface. The major difference between curli and CUP lies in its extracellular fiber growth assembly

(Hammar et al., 1996). After secretion of the CsgB subunit, CsgA precipitates, polymerizes on CsgB and adopts an insoluble structure related to amyloid fibers (Hammar et al., 1996).

1.4.2.1.3. Type IVB pili

One of the most studied adhesive structures of *S.* Typhi is the type IVB pilus encoded by the *pil* operon located on SPI-7. Although type IV pili also produce long and flexible structures on the bacterial cell surface, their mechanism of assembly strongly differs from the CUP and curli pathways as it requires many structural proteins and is an ATP-dependent process. First, PilS prepilins are translocated through the IM into the periplasm and a specific prepilin peptidase cleaves the N-terminal signal peptide (reviewed in Craig & Li, 2008). An integral IM protein mobilizes a specific ATPase from the cytoplasm which drives pilus assembly. An oligomeric channel called the secretin found in the OM allows the exit of the pilus at the cell surface of the bacteria. ATP hydrolysis moves the pilus out in the secretin pore allowing the recruitment of new prepilin subunits. Unlike CUP and Csg fimbriae, Type IV pili are still connected to the IM of the bacteria and can be retracted rapidly inside the bacteria.

1.4.2.2. Roles of fimbrial adhesins during typhoid fever

In most studies, *Salmonella* fimbriae are involved during intestinal colonization (Althouse et al., 2003; Chessa et al., 2009; Weening et al., 2005), or in biofilm formation (Boddicker et al., 2002; Ledeboer et al., 2006), although they can also be used during the systemic phase (Edwards et al., 2000; Lawley et al., 2006). Interestingly, each serovar of *Salmonella enterica* harbours a unique combination of fimbrial operons, probably to avoid cross-immunity between two serovars infecting the same host (Norris & Bäumler, 1999; Nuccio et al., 2011). As *S.* Typhi infects only humans, little is known regarding the conditions of expression or the implication of each fimbrial adhesin during the course of infection. While some clues may be found in the literature, there is still much work to be done. Three fimbrial systems are clustered within pathogenic islands: *tcf* (Typhi colonizing factor) and

saf (*Salmonella* atypical fimbriae) are found within SPI-6, while *sef* is in SPI-10 (Sabbagh et al., 2010).

Proteins expressed during infection were detected in blood of patients with typhoid fever (Charles et al., 2010; Harris et al., 2006; Hu et al., 2009). Interestingly, six proteins related to fimbrial adhesins led to the formation of antibodies after typhoid fever (TcfB, StbD, CsgG, CsgF, CsgE and BcfD). Since three proteins belonging to the thin aggregative fimbriae were identified, it suggests a strong production *in vivo* as well as an important role during infection. Csg implication during attachment to surfaces, bacterial autoaggregation and in biofilm formation is well known for *S.* Typhimurium and *E. coli* (Jonas et al., 2007). Nevertheless, a clear characterization of *csg* is needed for *S.* Typhi as there seem to be variations in expression between the different isolates (Römling et al., 2003; White et al., 2006). In *S.* Typhi, a strong expression of *csg* and *saf* fimbrial operons was observed inside human macrophages (Faucher et al., 2006).

The *bcf*, *sef*, *ste*, *stg* and *sth* fimbrial systems harbour pseudogenes that might disrupt the production of the corresponding fimbriae (Townsend et al., 2001). However, deletion of *stg* leads to reduced adhesion on epithelial cells as well as enhancement of the phagocytosis rate by macrophages (Forest et al., 2007). Furthermore, the presence of antibodies directed against BcfD is intriguing since the *bcfC* usher harbours two premature stop codons (Parkhill et al., 2001). The Bcf, Stb, Stc, Std and Sth fimbrial systems are required for the intestinal persistence of *S.* Typhimurium in mice, but their roles during the pathogenesis of *S.* Typhi still need to be evaluated (Weening et al., 2005). Sta and Tcf do not seem to be used for adhesion or invasion of non-polarized human epithelial cells while both are expressed at high NaCl concentrations (Bishop et al., 2008). Since these two fimbriae are found almost exclusively in the genome of serovars causing typhoid fever, they might be involved during the systemic phase or for the chronic carrier state (Nuccio et al., 2011). Although roles for Saf (Carnell et al., 2007; Lawley et al., 2006), Sef (Edwards et al., 2000) and Std fimbriae (Chessa et al., 2008; Weening et al., 2005) have been observed in other serovars of *Salmonella*, their true implication during typhoid fever needs to be investigated.

Type 1 fimbriae encoded by the *fim* operon are the best studied fimbrial adhesins and are frequently found in enteric bacteria. Fim are characterized by their mannose-sensitive binding properties, but their cell tropism seems to vary greatly between species and even between different strains of the same serovar (Thankavel et al., 1999). In *S.* Typhi, most clinical strains are fimbriated (*fim*+) and afimbriated strains are less adhesive and invasive than the fimbriated ones (Duguid et al., 1966; Satta et al., 1993). The ability of type 1 fimbriae to agglutinate yeast is abolished when the Vi capsule is expressed (Miyake et al., 1998). In *S.* Typhimurium, Fim appears to be the only fimbrial adhesin expressed in Luria-Bertani (LB) broth as confirmed by electron microscopy and flow cytometry (Duguid et al., 1966; Humphries et al., 2003). In *S.* Typhi, a complete deletion of *fim* also showed no evident fimbrial structures on the cell surface of the bacteria after growth in LB broth (Fig. 2).

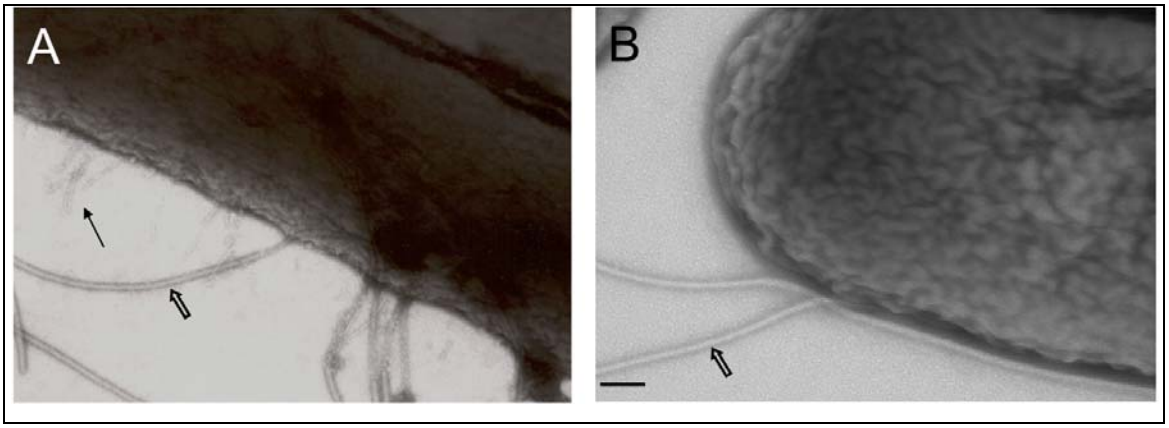

Figure 2. Surface observation of *S.* Typhi grown in LB broth by transmission electron microscopy. After negative staining with phosphotungstate 1%, fimbriae were observed at the cell surface of the wild-type ISP1820 strain (A), while no structure was observed when *fim* was deleted (B). Black arrow shows fimbria and the open arrows indicate flagella. Black bar = 100 nm.

Type IVB pili interact with the cystic fibrosis transmembrane conductance regulator (CFTR), a receptor upregulated and actively used by *S.* Typhi for its interaction with human

epithelial cells (Lyczak & Pier, 2002; Pier et al., 1998; Tsui et al., 2003). These pili can also mediate bacterial self-association in conditions found in the intestinal tract, probably by enhancing binding efficiency prior to cell invasion (Morris et al., 2003a; Morris et al., 2003b). A direct correlation was observed between the level of surface-exposed CFTR and the efficiency of invasion of *S*. Typhi through the intestinal barrier (Pier et al., 1998). This specific interaction can be blocked by the addition of prepilin pre-PilS in the cell culture medium or with monoclonal antibodies specific to the first extracellular domain of CFTR (Pier et al., 1998; Zhang et al., 2000). A piliated strain also adheres and invades human monocytes in a greater extent than a non-piliated strain and its expression can also increase IL-6 and NF-kappa B production in human monocytes by activating protein kinase C (Pan et al., 2005; F. Wang et al., 2005). Only a few other serovars, such as *S*. Paratyphi B and C, *S*. Heidelberg and *S*. *bongori* possess the genetic information coding for type IVB pili (Nuccio et al., 2011). Other functions could potentially be found in future studies as Type IV pili are also implicated in a variety of processes like biofilm formation, immune escape, DNA uptake and phage transduction in other pathogenic bacteria (reviewed in Craig & Li, 2008). These pili can also act as pistons, retracting subunits into the bacteria while it is still attached to a surface in a mechanism called "twitching motility" providing flagella-independent motility (reviewed in Mattick, 2002).

No genes related to fimbrial operons were found after a screening for mutants with a competitive disadvantage in humanized mice engrafted with hematopoietic stem cells (Libby et al., 2010). This result strongly suggests that fimbrial operons are mostly required during interaction with human epithelial cells, such as intestinal and gallbladder cells, that are absent from this mice model. Moreover, functional redundancy is often observed for fimbrial adhesins making it hard to evaluate their true contribution by single mutations. In order to understand the specific role played by each fimbrial system, our laboratory is currently creating a *S*. Typhi strain with deletions of all its fimbrial adhesins. This strain will greatly help to evaluate the global contribution of each fimbrial adhesins during association with eukaryotic cells.

1.4.3. Non fimbrial adhesins

1.4.3.1. Type 1 secretion systems

In *Salmonella*, some important surface structures are expressed by different mechanisms and can be classified as non fimbrial adhesins. In *S.* Typhi, there are two examples of adhesins secreted by a type I secretion system (T1SS) : SiiE and BapA. In T1SS, the secreted proteins directly pass through a channel formed between the IM and OM of the bacteria by the recognition of a signal at the C-terminus (China & Goffaux, 1999 as cited in Main-Hester et al., 2008). SPI-4, present in all *Salmonella* strains, encodes a T1SS responsible for the secretion of SiiE, the largest protein found in *Salmonella* (595 kDa) (Latasa et al., 2005; Main-Hester et al., 2008). Its cell surface expression requires the IM ATPase SiiF, the periplasmic adaptor SiiD and an outer membrane channel formed by SiiC (Gerlach et al., 2007). This adhesion system acts in a coordinated way with the T3SS of SPI-1 and is involved during the intestinal phase of infection (Gerlach et al., 2008). Previously annotated as two distinct ORFs in *S.* Typhi (STY4458-4459) (Parkhill et al., 2001), *siiE* harbours a premature stop codon probably rendering this large adhesin non-functional (Main-Hester et al., 2008; Morgan et al., 2004). An immunoblot done with antibodies directed against SiiE (STY4458) demonstrated the absence of production of SiiE in the whole cell proteins of *S.* Typhi further suggesting a lack of function in this serovar (Main-Hester et al., 2008). However, a transposon insertion in STY4458 showed a reduced competitive fitness in humanized mice, suggesting SiiE functionality and an uncharacterized role during interaction with hematopoietic cells (Libby et al., 2010).

A second T1SS is clustered within SPI-9 and secretes another large repetitive protein called BapA (biofilm-associated protein) due to its similarity with BapA of *Staphylococcus aureus*. Well described in *S.* Enteritidis, BapA is involved in bacterial autoaggregation strongly inducing biofilm formation and is also required during the interaction with the intestinal mucosa (Latasa et al., 2005). Its expression is under the control of CsgD, an important regulator also coordinating curli fimbriae and cellulose production needed for

biofilm production (Jonas et al., 2007). Again, solving the components required for biofilm formation by *S.* Typhi is crucial since *bcsC* (STY4184), essential for cellulose and biofilm production, is a pseudogene (Parkhill et al., 2001; Zogaj et al., 2001).

1.4.3.2. Type 5 secretion systems

Autotransported adhesins can be monomeric or trimeric and are considered as type 5 secretion systems. *S.* Typhi harbours two monomeric examples of autotransported adhesins, *shdA* (STY2755) found in the CS54 island and *misL* (STY4030) clustered in SPI-3, as well as one representative of a trimeric autotransporter called *sadA* (STY4105). An N-terminal signal sequence allows their translocation into the periplasm by the Sec general secretory pathway, then a β-domain found at the C-terminal end of the protein adopts a β-barrel conformation in the OM allowing secretion of the passenger domain into the extracellular space (reviewed in Nishimura et al., 2010). ShdA is widely distributed in *S. enterica* subspecies I and appears to be produced during typhoid fever despite the presence of a frameshifting sequence (Harris et al., 2006; Parkhill et al., 2001). Interestingly, ShdA and MisL can bind fibronectin in other serovars of *Salmonella* and are both considered as pseudogenes in *S.* Typhi (Dorsey et al., 2005; Kingsley et al., 2002). SadA harbours homology to the trimeric autotransporter adhesin YadA of *Yersinia enterocolitica*, a highly repetitive fibrous surface protein (Grosskinsky et al., 2007). YaiU (STY0405) encodes a fourth putative autotransported adhesin with no known role except that antibodies against the protein are produced during a typhoid fever (Harris et al., 2006).

1.4.3.3. Other adhesins

Besides fimbrial and afimbrial adhesins, other surface-exposed proteins can act as adhesins and mediate crucial roles during typhoid fever. One of the most hydrophobic proteins encoded in the *S.* Typhi chromosome, STY0351, was recently characterized in detail and might be used as a potential vaccine target. This cell-surface protein is a novel adhesin directly involved in the pathogenesis of *S.* Typhi by conferring strong binding to the laminin extracellular matrix (Ghosh et al., 2011) and is positively regulated by the PhoP-

PhoQ two-component system (Charles et al., 2009). It also possesses high immunogenic properties and STY0351-specific antibodies confer protection in a mouse model (Charles et al., 2010; Ghosh et al., 2011). PagC is another surface-exposed protein activated by the PhoP-PhoQ system that is produced and actively recognised by antibodies from patients having previously suffered from typhoid fever (Charles et al., 2010; Harris et al., 2006). Previously associated with survival within macrophages (Miller et al., 1989), PagC possesses serum resistance activity (Nishio et al., 2005) and can promote OM vesicle release in *S.* Typhimurium (Kitagawa et al., 2010), but none of these roles are confirmed yet for *S.* Typhi.

Multivalent adhesion molecules (MAM) are outer membrane proteins harbouring 6 or 7 mammalian cell entry domains and are widely found in pathogenic Gram-negative bacteria (Krachler et al., 2011). MAM mediates early interactions with different cell types by providing protein as well as lipid interactions with fibronectin and phosphatidic acid (Krachler et al., 2011). The specificity for certain cell types is thought to be provided by the other adhesins clustered throughout the genome of the bacteria. In *S.* Typhi, BLASTP analysis revealed that STY1980 harbours about 96% homology with MAM7 of the EPEC strain *E. coli* O127:H6 (Altschul et al., 1990) and could be implicated during the primary interactions with the intestinal mucosa.

1.4.4. Capsule and LPS

S. Typhi produces a group 1 exopolysaccharide known as the Vi antigen. Thus, *S.* Typhi is one of the few *Salmonella* serovars that get shielded by an extracellular polysaccharide layer constituting the Vi capsule. The Vi polysaccharide is a linear homopolymer of α(1→4)-2-acetamido-3-*O*-acetyl-2-deoxy-α-D-galacturonic acid (Heyns et al., 1959) and constitutes the major component of an injectable conjugated vaccine presently used against typhoid fever world-wide (World Health Organization, 2003). Vi has been involved in pathogenicity by evading the host innate immune system as it protects bacteria from phagocytosis and complement-mediated killing (Kossack et al., 1981). The *in vitro* masking of the OAg by the Vi antigen has been known for a long time (Felix & Pitt, 1934

as cited in Robbins & Robbins, 1984), prevents recognition by TLR-4, and limited C3 deposition to the cell surface (Looney & Steigbigel, 1986), which will lead to reduced clearance of the bacteria (Wilson et al., 2011). Vi is preferentially expressed at low osmolarity and early during infection of human macrophages or mice and will be downregulated with the progression of infection (Daigle et al., 2001; Faucher et al., 2006; Janis et al., 2011). The expression of Vi reduces invasion, probably by limiting the access of the T3SS-1 (Arricau et al., 1998; L. Zhao et al., 2001) or by masking other adhesion molecules including Fim. Vi is also important for surviving in macrophages (Hirose et al., 1997). Vi is tightly regulated by its own activator TviA (Hashimoto et al., 1996; Virlogeux et al., 1996), the two-component system OmpR-EnvZ (Pickard et al., 1994), the Rcs system (Arricau et al., 1998; Virlogeux et al., 1996) and repressed by RpoS (Santander et al., 2007).

Lipopolysaccharide (LPS) is the principal component of the outer membrane of Gram-negative bacteria and a major virulence determinant of many pathogens (Raetz & Whitfield, 2002). It is a glycolipid consisting in three structural regions covalently linked: (i) lipid A, also known as endotoxin, a hydrophobic anchor composed of acyl chains linked to phosphorylated *N*-acetylglucosamine; (ii) the inner and outer core composed of conserved oligosaccharides and; (iii) a variable polysaccharide chain or OAg. *Salmonella* OAg exhibits extensive composition and structural variation and has been divided into 46 O serogroups (Popoff et al., 2001). The O9 antigen of *S.* Typhi is characterized by the presence of a tyvelose residue. In response to acidified macrophage phagosomes, genes activated by the PhoP-PhoQ and PmrA-PmrB systems can modify the global structure of LPS and protect *Salmonella* from being killed by the immune system, notably by antimicrobial peptides (reviewed in Gunn, 2008). Heterogeneity in the length of the OAg repeats has been observed (P. Reeves, 1993) and is important for serum resistance and interaction with host cells (Bravo et al., 2011; Hoare et al., 2006; Hölzer et al., 2009). The *S.* Typhi OAg is essential for serum resistance but is not required for cell invasion (Hoare et al., 2006). Internalization of *S.* Typhi by epithelial cells involves the LPS core (Hoare et al., 2006) which acts as a ligand for CFTR (Lyczak et al., 2001; Pier et al., 1998). The *S.* Typhi

LPS core is involved in intracellular replication in macrophages (unpublished data), as observed with *S.* Typhimurium (Nagy et al., 2006; Zenk et al., 2009). *S.* Typhi does not have a bimodal distribution of OAg as it cannot produce very long OAg, consisting of more than 100 repeats of OAg units, because the major regulator Wzz (FepE) is non functional (Raetz & Whitfield, 2002). LPS biosynthesis involves many genes located in different clusters on the chromosome and may be controlled through several regulatory systems (P.R. Reeves et al., 1996). In *S.* Typhi, OAg expression is regulated by RfaH under the control of sigma factor RpoN (Bittner et al., 2002).

1.4.5. Secretion systems

1.4.5.1. Type 3 secretion systems

S. enterica harbours two distinct type 3 secretion systems (T3SSs) located on SPI-1 (T3SS-1) and SPI-2 (T3SS-2) that are crucial to its virulence along with a flagellar apparatus. T3SSs are complex molecular machines built from more than 20 different proteins, forming a structure similar to a molecular syringe (Kubori et al., 1998, Kimbrough & Miller, 2000 as cited in Sanowar et al., 2010). IM and OM rings are connected by a channel called the needle complex. These structures can inject many protein effectors directly from the bacterial cytoplasm to the cytoplasm of the eukaryotic cells, allowing a direct manipulation of host cellular pathways. The injection process is energized by specific cytoplasmic ATPase and direct contact with the eukaryotic cells is needed in order to activate secretion. Although T3SS are surface-exposed molecules, the lack of specific antibodies against the T3SS in the sera of convalescent patients of typhoid fever (Charles et al., 2010; Harris et al., 2006; Hu et al., 2009) might be a consequence of their tight regulation.

1.4.5.1.1. T3SS-1

In order to cause a systemic infection, *Salmonella* must first cross the intestinal epithelial barrier. Conditions found in the intestine, such as low oxygen tension and high osmolarity, are known to induce T3SS-1 of *Salmonella* by the HilA central regulator (Bajaj et al., 1996,

Galán & Curtiss, 1990 and Jones & Falkow, 1994 as cited in Altier, 2005). Injection of effectors secreted by the T3SS-1 mediates the invasion of non-phagocytic epithelial cells by *Salmonella* (Galán & Curtiss, 1989; Galán, 1999). Effectors interact with the actin cytoskeleton and induce membrane ruffles around the bacteria allowing its internalisation into epithelial cells. In *S.* Typhi, the contribution of the T3SS-1 during invasion of epithelial cells was confirmed with *invA*, *sipEBCDA* or *iagAB* (*hilAB*) mutants (Galán & Curtiss, 1991; Hermant et al., 1995; Miras et al., 1995). The T3SS-1 of *S.* Typhi may also play a role during the systemic phase of the infection (Haraga et al., 2008; Libby et al., 2010).

1.4.5.1.2. T3SS-2

After reaching the epithelial submucosa, *Salmonella* encounters and enters immune system cells like macrophages, dendritic cells and neutrophils. The intracellular environment of these cells promotes induction of the T3SS-2, which is regulated by the SsrA-SsrB two-component regulatory. Inside cells, bacteria are found in a *Salmonella*-containing vacuole (SCV) and inject T3SS-2 effectors to modify the SCV, alter host pathways and promote intracellular survival (Brumell et al., 2001; Waterman & Holden, 2003; Yu et al., 2004). Although *S.* Typhimurium absolutely requires the T3SS-2 for its intramacrophage survival (Cirillo et al., 1998; Hensel et al., 1998), a complete deletion of this system does not impair survival of *S.* Typhi in human macrophages (Forest et al., 2010). Nevertheless, *S.* Typhi T3SS-2 might be required for survival in other immune cells, as a mutant harbouring a transposon insertion in *ssrB* is disadvantaged in a humanized mouse model (Libby et al., 2010).

1.4.5.1.3. Flagella

The flagellar apparatus constitutes a third T3SS that is under the control of a highly organized transcriptional hierarchy involving three promoter classes with *flhDC* being the first activator (Kutsukake et al., 1990 and Karlinsey et al., 2000 as cited in Chevance & Hughes, 2008). In *Salmonella*, each cell harbours 6-8 peritrichous flagella built from more

than 25 different proteins (Harshey, 2011). The final structure is composed of a basal body, including a stationary and a moving rotor, an external hook and the filament comprised of flagellin (Harshey, 2011). Secretion of flagellin subunits and motility processes are powered by the proton motive force (Minamino & Namba, 2008 and Paul et al., 2008 as cited in Chevance & Hughes, 2008). Subspecies I, II, IIIa and IV of *S. enterica* are considered biphasic since they can alternatively express FliC or FljB major flagellar subunits in a mechanism known as phase variation (Lederberg & Iino, 1956; Simon et al., 1980). Most *S.* Typhi strains do not possess the *fljB* locus and are monophasic, but some isolates contain a 27 kb linear plasmid harbouring the *fljB*:z66 encoding for a novel flagellin (S. Baker et al., 2007; Frankel et al., 1989). Flagella normally contribute to the virulence through motility and chemotaxis (Macnab, 1999), but can also be implicated during biofilm formation (Crawford et al., 2010a). Flagellin can be detected by TLR-5 present at the cell surface of monocytes, dendritic cells and epithelial cells inducing proinflammatory and adaptive immune responses (Hayashi et al., 2001). In *S.* Typhi, TviA directly downregulates flagellar expression thereby avoiding its early recognition by the intestinal mucosa (Winter et al., 2008). Flagellar genes are involved in survival within macrophages or during the systemic phase of infection (Bäumler et al., 1994; Chan et al., 2005; Klumpp & Fuchs, 2007; Libby et al., 2010; Y. Zhao et al., 2002). Nevertheless, the real contribution of the flagellar apparatus is hard to evaluate since expression of the T3SS-1 is co-regulated with the flagella (Eichelberg & Galán, 2000; Saini et al., 2010). Interestingly, patients harbouring antibodies directed against flagella had uncomplicated typhoid fever, while prevalence of anti-outer membrane proteins (OMP) antibodies was associated with increased ileal perforation rates (Nambiar et al., 2009).

1.4.5.2. Type 6 secretion systems

Type 6 secretion systems are newly-discovered structures present in about 25% of sequenced Gram-negative bacterial genomes (Boyer et al., 2009). In *S. enterica* subsp. I, T6SS can be identified within SPI-6 (*S.* Typhi), SPI-19, SPI-20 or SPI-21 (Blondel et al., 2009). T6SS are contractile injection machinery harbouring strong similarities to the tail

sheath and spike of bacteriophages (Bönemann et al., 2010). These tubular structures can penetrate eukaryotic as well as prokaryotic membranes in a cell-contact dependant way in order to inject protein effectors. T6SS are often required within phagocytic cells (Ma et al., 2009; Pukatzki et al., 2009), but they can also be implicated in biofilm formation (Aschtgen et al., 2008; Enos-Berlage et al., 2005), colonization of the gastrointestinal tract (Blondel et al., 2010), quorum sensing (Weber et al., 2009) as well as in the delivery of toxins to other cells (Hood et al., 2010). Although *S.* Typhi harbours a pseudogene in *sciS*, a key component of its T6SS, as well as in *sciI*, the system is functional and its presence corresponds to an enhanced cytotoxicity toward epithelial cells (M. Wang et al., 2011). T6SS expression is regulated by RcsB, PmrA and Hfq (M. Wang et al., 2011). Its contribution during the interaction with hematopoietic cells should be further studied since a transposon insertion in two genes encoded within SPI-6 showed a competitive disadvantage in humanized mice (Libby et al., 2010).

Table 1. *S.* Typhi surface structures considered in this review and their roles in virulence.

Surface structure	**Role in virulence**	**Observed for *S.* Typhi**	**Observed in other serovars**
Bcf and Stb	Seroconversion Intestinal persistence in mice	Harris 2006; Hu 2009	 Weening 2005
Fim	Binds to mannose, adhesion and invasion of epithelial cells Biofilm	Satta 1993	Althouse 2003 Boddicker 2002
Stg	Adhesion to epithelial cells	Forest 2007	
Sth	Long-term infection of mice		Lawley 2006
Saf	Intestinal colonization of swine Long-term infection of mice		Carnell 2007 Lawley 2006
Sef	Interaction with macrophages		Edwards 2000
Stc	Intestinal persistence in mice		Weening 2005
Std	Binds to α(1,2)fucose Intestinal persistence in mice		Chessa 2009 Weening 2005
Tcf and YaiU	Seroconversion	Harris 2006	
Csg	Seroconversion Biofilm	Harris 2006	 Ledeboer 2006
SiiE	Adhesion to apical side of epithelial cells		Gerlach 2008

Surface structure	Role in virulence	Observed for *S.* Typhi	Observed in other serovars
SiiE	Colonization of the gastrointestinal tract		Blondel 2010
BapA	Interaction with intestinal mucosa, bacterial autoaggregation and biofilm		Latasa 2005
ShdA	Seroconversion Binds to fibronectin	Harris 2006	 Kingsley 2002
MisL	Intestinal colonization, binds to fibronectin		Dorsey 2005
Type IVB pili	Binds to CFTR, cellular invasion Bacterial self-association	Pan 2005; Pier 1998 Morris 2003b	 Morris 2003a
STY0351	Seroconversion Cell adhesion and binds to laminin	Charles 2010 Ghosh 2011	
PagC	Seroconversion Survival within macrophages Serum resistance OM vesicle release	Charles 2010; Harris 2006	 Miller 1989 Nishio 2005 Kitagawa 2010
Vi Capsule	Host immune system evasion Intramacrophage survival	Kossack 1981; Looney, 1986; Wilson 2011 Hirose 1997	
LPS	Binds to CFTR Antimicrobial peptides resistance Serum resistance Intramacrophage survival	Lyczak 2001 Baker 1999 Hoare 2006 Unpublished data	 Gunn 2008 Bravo 2008 Nagy 2006
T3SS-1	Effectors secretion and invasion of eukaryotic cells	Galán 1991; Hermant 1995	Galán 1989, 1999
T3SS-2	Effectors secretion and intramacrophage survival		Cirillo 1998; Hensel 1998
Flagella	Motility and chemotaxis Intramacrophage survival Biofilm formation	Liu 1988 Unpublished data	Macnab 1999 Bäumler 1994 Crawford 2010a
T6SS	Colonization of the gastrointestinal tract		Blondel 2010

1.4.7. Future perspectives

The multidrug-resistance observed for *S*. Typhi strains is of great concern since the total number of cases shows an increase of 38% during the last decade (Crump et al., 2004; Pang et al., 1998). There are two crucial lines of defence that should be improved in order to win the combat against typhoid fever: prevention and treatment. The best vaccine would be safe, given in a single dose, offering an efficient and long lasting immunity and remain stable at room temperature. Next generation vaccines have been recently tested in human trials (reviewed in Lindow et al., 2011). The expression of surface structures is tightly coordinated to avoid recognition by the immune system. Nevertheless, we have some clues regarding the structures recognized during typhoid fever (Charles et al., 2010; Harris et al., 2006; Hu et al., 2009). Since antibodies promote killing of *S*. Typhi (Lindow et al., 2011), a good approach to improve the efficiency of vaccines might be to create an avirulent strain expressing its immunogenic structures on inducible promoters inside antigen presenting cells (S. Wang et al., 2011).

Another strategy in the fight against *S*. Typhi should be the identification and treatment of the 1-5% infected individuals who become asymptomatic carriers (Crawford et al., 2010b; Parry et al., 2002). This task is complicated as antibiotherapy is often unsuccessful to remove biofilms found in the gallbladder, especially on gallstones, and surgical removal of the gallbladder is usually required but expensive (Crawford et al., 2010b; Prouty et al., 2002) Hence, efforts should be taken to understand the specific structures required for biofilm formation by *S*. Typhi in order to develop therapies to eliminate typhoid carriage.

Novel strategies are being developed to target surface structures implicated in bacterial pathogenesis as potential treatments (reviewed in Lynch & Wiener-Kronish, 2008). For example, pilicides are small compounds preventing interactions between the OM usher and chaperone-subunits complexes of type 1 pili, hence interfering with fimbrial biogenesis (Pinkner et al., 2006). Since most surface structures of *Salmonella* are expressed by the CUP, targeting the fimbrial ushers might be a useful method to eliminate colonisation and avoid the resulting antimicrobial resistance. Moreover, curlicides are able to interfere with

CsgA polymerization as well as type 1 fimbrial biogenesis resulting in the blocking of biofilm accumulation (Cegelski et al., 2009). Similarly, small-molecule inhibitors and inactivating antibodies can target binding or translocation of effectors by T3SS (Hudson et al., 2007; Neely et al., 2005; Nordfelth et al., 2005; Swietnicki et al., 2011). Targeting the capsule or LPS biosynthetic pathways might be a good approach to fight against *S*. Typhi since there is no corresponding enzyme in its human host (Cipolla et al., 2010; Goller & Seed, 2010).

Finally, understanding the role and function of *S.* Typhi surface proteins is primordial as these molecules are the first ones to directly interact with host components or cells, leading to a possibility for the development of new strategies to fight typhoid (see Table 1).

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1.4.9. References

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1.5. Biogenèse détaillée des fimbriae par la voie chaperonne/placier

Tel que décrit dans la section 1.4.7, il est primordial de caractériser les structures de surface de *S.* Typhi et de comprendre leurs mécanismes d'exportation dans le but d'améliorer l'efficacité des vaccins ou de développer de nouveaux traitements ciblés. Puisqu'il existe 12 représentants assemblés par la voie d'exportation appelée « chaperonne/placier » dans le génome de *S.* Typhi et que les fimbriae sont généralement impliqués dans les processus de pathogénicité, ils pourraient constituer une excellente cible thérapeutique. Il s'avère donc crucial de connaître les étapes requises lors de l'exportation des sous-unités fimbriaires à la surface de la bactérie. Il est à noter que toutes les informations contenues dans cette section proviennent de résultats observés avec des sytèmes fimbriaires présents chez *E. coli*. Néanmoins, les similarités observées dans la structure des opérons fimbriaires chez les bactéries à Gram négatif et l'homologie observée au niveau protéique (173) permettent d'extrapoler les observations obtenues avec *E. coli* aux autres systèmes fimbriaires.

Dans le système le plus simple, quatre protéines sont généralement traduites à partir d'un même opéron : la sous-unité mineure située à l'extrémité du fimbria, la sous-unité majeure formant le filament, une protéine chaperonne périplasmique, ainsi que le placier situé dans la membrane externe (Fig. 1.5). La première étape constitue la traduction des précurseurs protéiques arborant une séquence signale située en N-terminal d'environ 20 acides aminés (144). Cette séquence permet la reconnaissance et l'exportation du cytoplasme vers le périplasme par le complexe SecYEG situé dans la membrane interne (144). Les protéines sont sécrétées dans un état non replié et une peptidase enlève la séquence signale afin de former les protéines matures. L'interaction avec l'enzyme DsbA permet la formation des ponts disulfures et favorise le bon repliement du placier avant son insertion dans la membrane externe par un mécanisme impliquant la chaperonne périplasmique SurA, ainsi que le complexe BAM (108, 183).

Dans le périplasme, les sous-unités fimbriaires adoptent une conformation comparable à une immunoglobuline manquant le 7^{e} brin β normalement retrouvé en C-terminal suite au repliement (35, 219, 220). Cette conformation expose l'intérieur hydrophobe de la sous-unité et peut être complétée par un brin provenant de la chaperonne dans un mécanisme appelé « complémentation du brin donneur » (35, 219). L'interaction spécifique entre la chaperonne et ses sous-unités assure leur bon repliement, empêche les appariements périplasmiques précoces et les protège de la dégradation (10, 136, 237).

Par la suite, ce complexe interagit avec le placier, un tonneau β inséré dans la membrane externe. La délétion du placier d'un opéron fimbriaire provoque la rétention du complexe chaperonne/sous-unité dans le périplasme, empêchant l'exportation et l'assemblage fimbriaire à la surface (123, 172, 256). La force d'affinité entre le placier et ses sous-unités dicte l'ordre d'assemblage de celles-ci : l'extrémité s'incorpore en premier, suivi des sous-unités majeures qui forment la structure principale du filament (Fig. 1.5) (54, 222). L'incorporation des sous-unités se fait par la base du fimbria (221). Chacune des sous-unités majeures possède une extension N-terminale qui déplace le brin β de la chaperonne et complète le repliement de la sous-unité incorporée précédemment dans un mécanisme appelé « échange du brin donneur » (219). La première sous-unité incorporée dans le filament ne contient pas cette extension, mais possède à la place un domaine de liaison aux récepteurs (221).

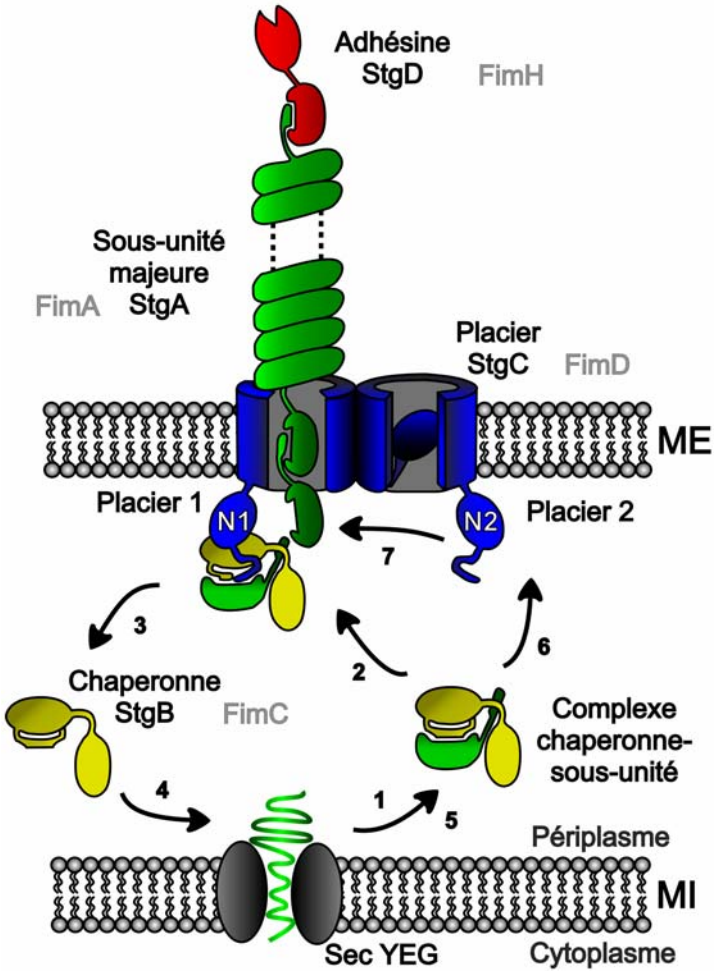

Figure 1.5. Schéma démontrant la biogenèse des fimbriae par la voie chaperonne/placier chez les bactéries à Gram négatif. 1) Suite à la sécrétion dans le périplasme par le système SecYEG, les sous-unités sont repliées par la chaperonne périplasmique. 2) Le complexe interagit avec le domaine N-terminal d'un de ses placiers. 3 à 6) La chaperonne est relâchée, puis réutilisée pour amener d'autres sous-unités aux placiers. 7) Un des deux placiers est fermé par son bouchon, mais apporte des sous-unités à l'autre placier utilisé pour l'assemblage du fimbria. Les protéines correspondantes retrouvées dans l'assemblage du fimbria de type 1 d'*E. coli* sont identifiées en gris, sauf les sous-unités mineures FimG et FimH qui n'ont pas d'homologue correspondant dans Stg.

Le placier catalyse l'assemblage fimbriaire et constitue le pore utilisé pour la sécrétion des sous-unités (170, 172, 208). La cristallographie récente du placier FimC retrouvé chez le fimbria de type 1 a permis d'observer les différents domaines impliqués dans le processus : une boucle en N-terminal, suivie de 6 feuillets β antiparallèles, un bouchon, les feuillets β 7 à 24 et deux domaines C-terminaux (Fig.1.6A) (194). La boucle située du côté N-terminal permet le recrutement et la liaison des complexes chaperonne/sous-unité (Fig.1.6B) (165, 169, 171). Les domaines situés en C-terminal sont essentiels pour effectuer la suite des évènements et permettent l'ancrage du fimbria en restant associés avec le dernier complexe chaperonne/sous-unité incorporé (70, 194, 234, 246). Durant la biogenèse, des dimères de placiers travaillent de façon coopérative pour le recrutement des sous-unités et l'assemblage d'un fimbria : un placier permet l'exportation des sous-unités à la surface bactérienne, alors que l'autre placier demeure fermé par son bouchon (132, 133, 145, 208). Puisqu'il n'a pas d'énergie disponible dans le périplasme, l'assemblage fimbriaire est un processus qui s'auto-alimente (108). En fait, la chaperonne replie les sous-unités dans une conformation activée qui relâche l'énergie lors de l'incorporation dans le fimbria en adoptant un repliement plus compact (220, 274).

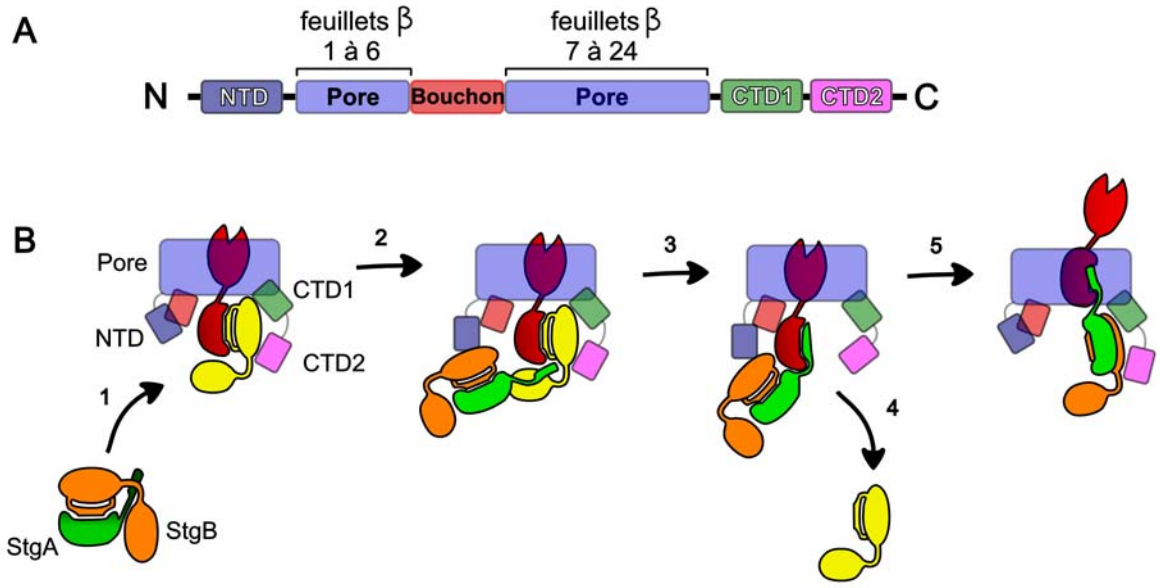

Figure 1.6. Détails moléculaires concernant le placier FimD d'*E. coli*. A) La protéine mature du placier FimD renferme un domaine N-terminal (NTD), suivi des 6 premiers feuillets β, un domaine constituant le bouchon, les feuillets β 7 à 24, ainsi que deux domaines C-terminaux (CTD1, CTD2). B) 1- Le premier complexe chaperonne/sous-unité majeure se forme par la « complémentation du brin donneur ». 2- Celui-ci interagit avec le domaine NTD du placier. 3- L'extension N-terminale de la sous-unité complète le repliement de l'adhésine dans un mécanisme appelé « échange du brin donneur ». 4- Ceci mène à la dissociation de la première chaperonne (jaune). 5- La deuxième chaperonne (orange) liée à la sous-unité majeure interagit avec les domaines C-terminaux du placier et pousse les sous-unités à travers le pore. Figure inspirée de (194).

1.6. Régulation de l'expression fimbriaire

Puisque les fimbriae peuvent être facilement reconnus par le système immunitaire, plusieurs mécanismes de régulation permettent leur expression dans des conditions environnementales spécifiques (36). Chez *Salmonella* et *E. coli*, la majorité des fimbriae ne sont pas exprimés dans les milieux de culture conventionnels, tel que le LB, démontrant l'efficacité de leur régulation (105, 139). Seul le fimbria de type 1 est facilement détectable

chez ces deux bactéries après croissance dans le milieu LB (voir la Figure 2 de la section 1.4.2.2) (105, 127).

Le premier niveau de régulation survient au niveau de la transcription et peut se produire de différentes façons. La liaison de protéines régulatrices directement sur les séquences promotrices des opérons fimbriaires constitue le moyen de régulation le plus fréquemment observé. Pour certains opérons fimbriaires, les gènes codant pour les régulateurs sont situés à proximité des gènes de biosynthèse, ce qui facilite grandement leur identification. Par exemple, le fimbria de type 1 de *S.* Typhimurium possède deux régulateurs positifs, FimY et FimZ, ainsi qu'un régulateur négatif FimW, dont les gènes sont retrouvés directement en aval de l'opéron *fim* (249, 250, 270). Plusieurs protéines impliquées dans la régulation du métabolisme, comme Lrp (*Leucine-responsive regulatory protein*) et Crp (*Cyclic AMP receptor protein*), contrôlent aussi la transcription des fimbriae (6, 149, 157).

Les protéines structurales impliquées dans la topologie de l'ADN, telles H-NS, IHF et Fis sont souvent associées à la régulation fimbriaire (36). Ces protéines sont capables d'influencer la courbure de l'ADN de façon locale et agissent généralement sur l'initiation de la transcription (55). Chez *E. coli* K-12, la protéine H-NS est reconnue comme étant un facteur qui inhibe plusieurs opérons fimbriaires à la fois; une mutation de H-NS induit l'expression de plusieurs fimbriae dans un milieu LB (127). Cette régulation négative par H-NS a été confirmée pour les fimbriae Csg et Fim de *S.* Typhimurium (80, 149). IHF agit en général de façon positive sur l'expression fimbriaire, par exemple en empêchant la liaison de H-NS ou en repliant l'ADN dans une conformation favorisant la liaison de l'ARN polymérase (39, 80, 177). L'activité de Fis est davantage versatile que H-NS et IHF. En effet, Fis peut agir en tant que répresseur de l'expression fimbriaire (82, 214), mais constituer un activateur essentiel lors de la formation de biofilms (230). Le surenroulement de l'ADN influence de façon globale la transcription du génome bactérien (180), mais il peut aussi influer sur la transcription des fimbriae (39). Le surenroulement négatif global est principalement régulé par la gyrase à ADN, alors que la topoisomérase I relâche le

surenroulement négatif local introduit par le mouvement de la polymérase à ARN (137). La transcription fimbriaire peut être influencée par ces deux phénomènes à la fois (245).

Certains fimbriae sont régulés par la variation de phase, un mécanisme permettant d'alterner entre un état fimbriaire et afimbriaire. La transcription des gènes de biosynthèse du fimbria de type 1 d'*E. coli* dépend spécifiquement de l'orientation du promoteur de *fimA* (61). Cette régulation requiert l'action des recombinases site-spécifique FimB et FimE et s'effectue grâce à la présence de deux séquences d'ADN de 9 pb inversées, situées en amont et en aval du promoteur de *fimA* (76, 122, 125).

Une autre façon pour réguler la transcription des fimbriae s'effectue au niveau de la méthylation de l'ADN. L'exemple classique pour ce type de régulation est le fimbria Pap d'*E. coli*, mais les fimbriae Pef et Std de *S.* Typhimurium sont aussi sous le contrôle de la méthylation de l'ADN (9, 19, 166). L'expression du fimbria Pap provient de la compétition exercée entre la liaison de Lrp, du régulateur PapI et de l'activité de la méthylase à ADN Dam au niveau de deux séquences GATC retrouvées en amont du promoteur de *pap* (93).

Récemment, la concentration intracellulaire de di-GMP cyclique (di-GMPc) a été reconnue comme étant un facteur influençant l'expression de certains fimbriae. Le di-GMPc est un messager secondaire fortement synthétisé lorsqu'il y a une carence en acides aminés par l'action d'une diguanilate cyclase possédant un domaine GGDEF. Son hydrolyse est effectuée par l'action d'une phosphodiestérase renfermant un domaine EAL. Lorsqu'il y a une forte concentration en di-GMPc, le développement de biofilms est favorisé, ainsi que l'expression de fimbriae. Les fimbria Csg de *Salmonella* et d'*E. coli* sont sensibles à la quantité de di-GMPc (113, 235).

En plus de tous ces moyens de régulation transcriptionnelle, l'obtention d'une stochiométrie parfaite au niveau des différentes sous-unités fimbriaires est assurée par la différence dans la stabilité de l'ARNm, l'affinité des sites de liaison du ribosome et le biais d'usage des codons (124). Par exemple, l'expression des trois régulateurs du fimbria de type 1 de *S.* Typhimurium est régulée au niveau traductionnel : *fimW*, *fimY* et *fimZ*

renferment tous les trois des codons d'arginine rares qui sont spécifiquement reconnus par l'ARNt *fimU* situé en 5' de *fimW* (241).

Récemment, la régulation croisée entre deux opérons fimbriaires a été observée : la protéine PapB utilisée pour l'expression du fimbria Pap peut inhiber l'activité de la recombinase FimB et empêcher l'expression des fimbriae de type 1 chez *E. coli* (97). Cette découverte suggère qu'il existe des niveaux de régulation très complexes encore inconnus pour l'expression fimbriaire. Encore une fois, la majorité des études de régulation fimbriaire ont été effectuées chez le sérovar Typhimurium, mais peuvent être utilisées lorsqu'il s'agit de fimbriae retrouvés chez *S.* Typhi. Il faut néanmoins effectuer la recherche directement avec le sérovar Typhi pour comprendre la régulation de ses cinq fimbriae absents du génome de *S.* Typhimurium (*sef*, *sta*, *ste*, *stg*, *tcf*).

1.7. Contexte de l'étude, hypothèse et objectifs

Le nombre d'infections causées par *S.* Typhi a considérablement augmenté dans la dernière décennie (42, 184). La présence de souches résistantes à tous les antibiotiques disponibles et de porteurs asymptomatiques constituent une menace mondiale, même pour les pays développés (48). Il est important de découvrir les facteurs de virulence impliqués dans la pathogenèse de *S.* Typhi, dans le but d'améliorer le vaccin, de trouver de nouvelles cibles ou des alternatives pour les antibiotiques. La majorité des études concernant la fièvre typhoïde proviennent d'un modèle d'infections de souris par *S.* Typhimurium qui a été très utile par le passé pour déterminer les facteurs de virulence nécessaires pour une infection productive. Cependant, *S.* Typhimurium cause une gastro-entérite chez l'humain et plusieurs différences génétiques sont notables lorsque son génome est comparé avec celui du sérovar Typhi. Ainsi, il existe très peu d'informations concernant le rôle des gènes retrouvés uniquement chez *S.* Typhi dans sa pathogenèse. Cette implication est difficile à évaluer puisqu'il infecte seulement les humains.

Afin de produire une infection systémique, *S.* Typhi doit réussir à survivre à l'intérieur des macrophages humains (57, 58). Dans une étude précédente, notre laboratoire a identifié des gènes transcrits à l'intérieur de macrophages humains par *S.* Typhi et qui sont absents du génome de *S.* Typhimurium (65). Cette approche a permis l'identification de l'opéron fimbriaire *stgABCD* comme étant unique à *S.* Typhi. Une caractéristique intéressante de cet opéron est la présence prématurée d'un codon d'arrêt TAA au 171^{e} codon du gène *stgC*. Celui-ci code pour le placier, la protéine située dans la membrane externe, qui est responsable de l'exportation des sous-unités fimbriaires StgA et StgD pour former un fimbria complet. En théorie, la terminaison prématurée lors de la traduction de *stgC* devrait empêcher sa fonctionnalité et suggère l'absence totale de production de fimbriae Stg (253). En ce sens, il a été suggéré que la présence élevée de pseudogènes dans le génome du sérovar Typhi suggère une perte complète de fonction de ceux-ci (100, 253). D'autres études ont cependant démontré que le placier *lpf1CC'* d'*E. coli* O157:H7 renfermant un codon d'arrêt prématuré peut produire des fimbriae fonctionnels, mais la preuve de la nécessité du placier pseudogène n'a pas été effectuée (68, 112, 251).

L'hypothèse de ce travail est que l'opéron fimbriaire *stg* s'avère fonctionnel, malgré la présence d'un codon de terminaison prématuré dans le gène *stgC* codant pour le placier.

Pour vérifier cette hypothèse, les objectifs ont été de :

1) évaluer la contribution de *stg* lors de l'interaction de *S.*Typhi avec les cellules humaines
2) déterminer le mécanisme moléculaire permettant l'assemblage des fimbriae *stg*

Pour répondre au premier objectif, une fusion transcriptionnelle entre le promoteur de l'opéron *stg* et le gène rapporteur *lacZ* a été effectuée et testée dans diverses conditions de croissance. Une mutation complète de *stgABCD* a été effectuée par échange allélique chez *S.* Typhi. L'opéron *stg* a été cloné dans un plasmide qui a été introduit dans une souche afimbriaire d'*E. coli*, chez *S.* Typhimurium, ainsi que dans le mutant de *S.* Typhi. L'effet de *stg* dans la virulence a été évalué par des infections de cellules intestinales et de

macrophages humains avec les différentes souches. Les résultats du premier objectif sont présentés dans le Chapitre 2 de cette thèse.

La seconde partie consistait à déterminer si les fimbriae Stg sont assemblés par leur propre placier StgC, qui renferme un codon d'arrêt prématuré, ou s'il s'agit d'un phénomène de complémentation par un autre placier. Des délétions non-polaires ont été introduites dans *stgA*, *stgC* et *stgD*, puis le codon d'arrêt a été modifié pour un codon TAT qui introduit une tyrosine. Ces différentes versions de l'opéron ont été clonées sur un vecteur inductible à l'arabinose, puis transformées dans une souche afimbriaire d'*E. coli* et un anticorps polyclonal dirigé contre la sous-unité StgD a été produit. L'expression à la surface de StgD chez ces différents mutants a été vérifiée par Western et par microscopie électronique à transmission. Une étiquette de 6-histidines a été insérée dans la partie C-terminale de StgC pour vérifier sa traduction et la protéine StgC a été envoyée au séquençage. Une fusion traductionnelle a été réalisée entre *stgC* et la protéine verte fluorescente (GFP) pour évaluer la traduction chez *S*. Typhi. Les résultats de la deuxième partie sont présentés dans le Chapitre 3 de cette thèse.

Préface au Chapitre 2

La première étape pour vérifier la fonctionnalité de l'opéron *stg*, retrouvé uniquement chez *S.* Typhi, consistait à vérifier son niveau d'expression dans différentes conditions de croissance, à l'aide d'une fusion transcriptionnelle entre le promoteur de *stgA* et le gène rapporteur *lacZ*. De plus, le rôle de l'opéron fimbriaire *stg* a été évalué lors de l'interaction avec les cellules intestinales et des macrophages humains. Cet article a été soumis à un processus de révision par les pairs et a été publié dans le journal « Infection and Immunity ».

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Contribution des auteurs :

Le projet a été élaboré par France Daigle. J'ai réalisé les diverses expérimentations avec l'aide de Sébastien P. Faucher et Katherine Poirier. Sébastien Houle et Charles M. Dozois ont effectué de la microscopie électronique et des Westerns blots pour le projet. J'ai procédé à l'analyse des résultats, à la création des figures et des tableaux. Le manuscrit a été écrit par France Daigle et tous les coauteurs ont participé à sa révision. Les fonds nécessaires au projet ont été fournis par France Daigle.

CHAPITRE 2, 2e article

Contribution of the *stg* fimbrial operon of *Salmonella enterica* serovar Typhi during interaction with human cells

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Running title: *stg* fimbrial operon of Typhi

Keys Words: *Salmonella*, fimbriae, Stg, adhesion, pathogenesis

ABSTRACT

Salmonella serovars contain a wide variety of putative fimbrial systems that may contribute to colonization of specific niches. *Salmonella enterica* serovar Typhi is the etiologic agent of typhoid fever and is a pathogen specific to humans. In a previous study, we identified a gene, STY3920 (*stgC*), encoding the predicted usher of the *stg* fimbrial operon, that was expressed by serovar Typhi during infection of human macrophages. The *stg* genes are located in the *glmS-pstS* intergenic region in serovar Typhi and certain *Escherichia coli* strains, but they are absent in other *S. enterica* serovars. We cloned the *stg* fimbrial operon into a nonfimbriated *E. coli* K-12 strain and into *S. enterica* serovar Typhimurium. We demonstrated that the *stg* fimbrial operon contributed to increased adherence to human epithelial cells. Transcriptional fusion assays with serovar Typhi suggested that *stg* is preferentially expressed in minimal medium. Deletion of *stg* reduced adherence of serovar Typhi to epithelial cells. However, deletion of *stg* increased uptake of serovar Typhi by human macrophages, and overexpression of *stg* in serovar Typhi and serovar Typhimurium strains reduced phagocytosis by human macrophages. These strains survived inside macrophages as well as the wild-type parent. Although the *stgC* gene contains a premature stop codon that disrupts the expected open reading frame encoding the usher and is therefore considered a pseudogene, our results show that the *stg* operon may encode a functional fimbria. Thus, this serovar Typhi-specific fimbrial operon contributes to interactions with host cells, and further characterization is important for understanding the role of the *stg* fimbrial cluster in typhoid fever pathogenesis.

INTRODUCTION

The genus *Salmonella* is composed of two species, *Salmonella bongori* and *Salmonella enterica*. *S. enterica* comprises more than 2,400 serovars (11) and has been divided into seven subspecies (19). Subspecies I contains *S. enterica* serovars Typhi and Typhimurium and most of the other serovars that cause diseases in humans and other warm-blooded animals. Some serovars, such as serovar Typhimurium, cause disease in a variety of animals, whereas other serovars, such as Typhi, cause disease in only one or a few species. Serovar Typhi is a human-specific pathogen and the etiologic agent of typhoid fever, a systemic disease, whereas serovar Typhimurium causes localized gastroenteritis in most cases of human infection. In spite of a high degree of genome homology (>90%) between serovars Typhi and Typhimurium (22, 29), the difference in the types of diseases that these serovars cause in humans, systemic and localized, respectively, suggests that one difference between these pathovars might be in the way that these closely related pathogens interact with host cells. Each of these serovars might produce or secrete distinct molecules that contribute to differences in tissue tropism. The genomes of *Salmonella* serovars Typhi and Typhimurium were completed and compared previously (22, 29). The serovar Typhi strain CT18 genome contains 601 genes located in 82 unique genomic regions that are absent from the serovar Typhimurium strain LT-2 genome (29). Thus, it is likely that serovar Typhi possesses unique genetic information that may be important for systemic spread and survival in the human host. The largest unique region in serovar Typhi is 134 kb long and was designated *Salmonella* pathogenicity island 7 (SPI-7). SPI-7 harbors the *viaB* locus encoding the Vi antigen, which is used in the current conjugated vaccine (17). Vi is a polysaccharide capsule involved in preventing interleukin-8 production, thus reducing neutrophil influx in the intestine (31, 33). The *pil* genes coding for type IV pili facilitate bacterial entry into human epithelial cells and are also located on SPI-7 (43).

After ingestion, serovar Typhi is transported to the intestinal lumen, where it adheres to and invades the small intestine. Bacteria are taken up by mononuclear cells in the intestinal

lymphoid tissue, drain into the general circulation, and spread to the spleen and liver. After replication, a large number of bacteria are released into the bloodstream, which coincides with the onset of typhoid fever symptoms. In chronic carriers, bacteria can persist in the mesenteric lymph nodes, bone marrow, spleen, and gall bladder for the life of the patient. Many virulence factors may be needed and expressed during the course of infection.

Adhesion to host cells and mucosal surfaces is often considered an essential step because it allows bacteria to initiate colonization. Fimbriae or pili and other surface molecules mediate adherence via specific receptors on host cell surfaces. Genes encoding a wide variety of putative fimbriae are present in *Salmonella* serovars, but only a few *Salmonella* fimbriae have been characterized so far. These putative fimbriae may confer different binding specificities required at different steps of the infection and may be involved in host adaptation, by conferring the ability to bind to specific host cells. The genome sequence of serovar Typhi contains 13 putative operons corresponding to fimbrial gene sequences, designated *bcf, csg (agf), fim, saf, sef, sta, stb, stc, std, ste, stg, sth*, and *tcf*, as well as *pil* coding for the type IV pili (29). Five of these operons, *sef, sta, ste, stg*, and *tcf*, and the type IV pili were not detected in serovar Typhimurium (29). In a previous study, we determined that STY3920 (*stgC*), a gene encoding the usher of the putative *stg* fimbrial operon, is absent in serovar Typhimurium and is expressed by serovar Typhi during infection of human macrophages (6). *stgC* contains a premature stop codon that disrupts the predicted open reading frame (ORF) encoding the usher, and is therefore considered a pseudogene. As similar fimbrial clusters in *Escherichia coli* also contain genes with premature stop codons and have functional roles (7, 14, 26, 37), we hypothesized that the *stg* operon may encode functional fimbriae that contribute to the interaction of serovar Typhi with human cells. In this study, we cloned and characterized the *stg* fimbrial operon and demonstrated its role in adhesion to epithelial cells and phagocytosis by macrophages.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in Luria-Bertani (LB) broth at 37°C, unless indicated otherwise. When required, antibiotics, amino acids, or supplements were added at the following concentrations: kanamycin, ampicillin, and diaminopimelic acid (DAP), 50 μg/ml; chloramphenicol, 34 μg/ml; and tryptophan, cysteine, and arginine, 22 μg/ml. Transformation of bacterial strains was routinely done by using the calcium/manganese-based or electroporation methods as described previously (27).

Cloning of the *stg* fimbrial operon. The *stg* operon was amplified from genomic DNA of strain ISP1820 using the Elongase enzyme mixture (Invitrogen) with primer StgA-F (5'CGGGATCCGAGATGAGAATAACGGAATA-3') containing a BamHI restriction site (underlined) and primer StgD-R (5'GCTCTAGACATTGATATGACTTATTTTG-3') containing an XbaI restriction site (underlined). The 5-kb PCR product was purified and cloned into vector pCR2.1 using a TOPOXL PCR cloning kit (Invitrogen), resulting in plasmid pSIF018. The XbaI-HindIII fragment was subcloned into low-copy-number vector pWSK29 at the same restriction sites, resulting in plasmid pSIF026. The different constructs were transformed into the nonfimbriated *E. coli* K-12 Δ*fim* mutant strain ORN172 (42) or into *S. enterica* serovar Typhimurium and Typhi strains.

Adherence to human epithelial cells. The ability of *E. coli* strain ORN172 containing the *stg* operon (pSIF018) or only the vector (pCR2.1) to adhere to human epithelial cells (INT-407) was assessed. A total of 2.5×10^5 cells grown in minimal essential medium (Wysent) supplemented with 10% heat-inactivated fetal calf serum (Wysent) and 25 mM HEPES (Wysent) were seeded in 24-well tissue culture plates 24 h before the adherence assays. One hour before infection, cells were washed three times with prewarmed phosphate-

buffered saline (PBS) (pH 7.4), and fresh complete medium was added to each well. Bacteria were grown overnight on LB medium plates and were resuspended in PBS to an optical density at 600 nm (OD_{600}) of 1.5 (~ 1.5×10^9 CFU/ml). Approximately 2.5×10^7 CFU was added to each well (multiplicity of infection [MOI], 100). The 24-well plates were then centrifuged at 1,000 × *g* for 5 min to synchronize infection, incubated at 37°C in 5% CO_2 for 90 min, and rinsed three times with PBS. PBS-0.1% deoxycholic acid sodium salt was added to each well, and samples were diluted and spread on LB medium plates for enumeration by viable colony counting. The results were expressed as the percentage of the initial inoculum. Statistical differences were assessed using Student's *t* test.

A similar protocol was used to test adherence of *Salmonella* and/or the isogenic *stg* mutant strains to INT-407 cells, except that bacteria were grown overnight without shaking in LB medium containing 0.3 M NaCl and an MOI of 20 was used. When indicated below, an additional 90-min incubation with 100 μg/ml gentamicin to kill extracellular bacteria was performed in order to assess the invasion level.

Generation of a single copy *stgA-lacZ* transcriptional fusion and β-galactosidase assay. The *stgA* promoter region was amplified using the Elongase enzyme mixture (Invitrogen) and the following primers: StgA-F and StgA-R (5'AACTGCAGCCAGCAAATGCCGTTTTGTT3'). The PCR product was cloned into vector pCR2.1 using a TOPOXL PCR cloning kit (Invitrogen), resulting in plasmid pSIF016. A 530-bp fragment digested with XbaI and SpeI was purified and ligated to pFUSE digested with XbaI (2), resulting in plasmid pSIF020. Plasmid pSIF020 was confirmed to contain the *stgA* promoter in the correct orientation for *lacZ* fusion. To generate a single copy of the P*stgA-lacZ* fusion in serovar Typhi, pSIF020 was transferred by conjugation and integrated into the genome by homologous recombination as described previously (2, 3). A strain carrying a single integrated copy of P*stgA-lacZ* in ISP1820 was designated DEF068. The expression of *stg* was evaluated by β-galactosidase assays of the reporter strain DEF068 grown in different conditions. β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside as described previously (23).

Construction of a serovar Typhi strain with an *stg* deletion. A suicide vector for deletion of the *stg* fimbrial operon (STY3918 to STY3922) was constructed as follows. A 530-bp fragment of the 5' end of *stgA* was generated by PCR using primers StgA-F and StgA-R, and a 482-bp fragment of the 3' end of *stgD* was generated by PCR using primers StgD-F (5'AACTGCAGGCCGCAGAGCTGTGAAAATG3') and StgD-R. These two fragments were ligated and cloned into the XbaI and BamHI sites of pMEG-375 (15). A resulting suicide vector containing the *stgA'-stgD'* fragment (pSIF004) was used for allelic replacement of the *stg* region. The pSIF004 suicide vector was conjugated from *E. coli* MGN-617 to serovar Typhi strain ISP1820 by overnight plate mating on LB medium with DAP. Transconjugants were selected by growth on LB medium plates containing chloramphenicol without DAP. Selection for double-crossover allele replacement was performed by *sacB* counterselection on LB agar plates without NaCl containing 5% sucrose (16). Isogenic strain DEF004 has a deletion of the *stg* region resulting from a double crossover, as determined by the absence of resistance to ampicillin and chloramphenicol encoded on the suicide vector, and the expected *stg* deletion, as confirmed by PCR (data not shown).

Bacterial survival in human macrophages. The human monocyte cell lines THP-1 (= ATCC TIB-202) and U937 (= ATCC CRL 1593) were maintained in RPMI 1640 (Invitrogen) containing 10% fetal calf serum, 25 mM HEPES, 2 mM L-glutamine, 1% minimal essential medium nonessential amino acids (Wisent), and 1 mM sodium pyruvate (Sigma). Stock cultures of these cells were maintained as monocyte-like, nonadherent cells at 37°C in an atmosphere containing 5% CO_2. Before infection, cells were differentiated by addition of 10^{-7} M phorbol 12-myristate 13-acetate (Sigma) for 24 to 72 h. For macrophage infection assays, cells were seeded at a concentration of 5×10^5 cells per well in 24-well tissue culture dishes. Bacteria grown overnight at 37°C in static conditions were added to a cell monolayer at an MOI of 10 and centrifuged for 5 min at 1,000 × *g* to synchronize phagocytosis. After incubation for 20 min at 37°C (zero time), the infected cells were washed three times with prewarmed PBS and incubated with supplemented medium as

described above containing 100 μg/ml of gentamicin to kill extracellular bacteria. The infected monolayers were either lysed from the tissue culture dishes by addition of 0.1% deoxycholic acid sodium salt in PBS or incubated further. After lysis the number of surviving bacteria was determined by bacterial plate counting (CFU). The level of phagocytosis was expressed as a percentage of the initial inoculum. The survival rate was expressed as a percentage determined by comparing the number of intracellular bacteria with the number at the previous time.

Statistical differences were assessed using Student's *t* test. Where indicated, the macrophages were incubated 1 hr prior to infection with 1 μg/ml of cytochalasin D (Sigma) to inhibit bacterial uptake as described previously (32). The level of cytochalasin D was maintained throughout the infection.

RESULTS

***stg* fimbrial operon.** The *stg* fimbrial cluster has a G+C content of 49% and is a member of a distinct group of related fimbrial genes that are located in the *glmS-pstS* intergenic region (21, 39). In the sequenced genomes of *S. enterica* (including unfinished genomes) this fimbrial gene cluster has been identified only in serovar Typhi. Moreover, *stg* sequences were not detected by comparative genomic hybridization in the genomes of 140 strains belonging to many serovars of subspecies I (30, M. McClelland, personal communication). The previously described distribution of *stg* determined by Southern blotting may therefore represent cross-hybridization with other less homologous fimbrial genes (39). However, a putative fimbrial gene inserted in the *glmS-pstS* region in *S. bongori* belongs to the Stg group, and its product exhibits the highest level of identity to the predicted *stg* fimbrial gene products of serovar Typhi (Table 2). The genes encoding a number of fimbrial systems in pathogenic *E. coli* are also inserted in the *glmS-pstS* region and belong to the Stg group; these systems include the Stg (21), Lpf_{O113} (5) and Lpf2 (O-island 154) (38) systems. In addition, Lpf and related fimbriae encoded in the *yhjX-yhjW* region in *Salmonella* and *E. coli* (36, 37) exhibit some identity to the predicted *stg* gene products of serovar Typhi, but less identity than other fimbriae belonging to the Stg group (Table 2). The serovar Typhi *stg* fimbrial cluster contains five ORFs designated *stgABCC'D* as *stgC* is a predicted pseudogene and contains a premature stop codon. The *stgC* ORF may code for a 170-amino-acids (aa) protein, and a second ORF designated *stgC'* may code for a 605-aa protein. The *stgC* stop codon is present in the *stgC* sequence of serovar Typhi strain ISP1820 (data not shown), as well as in the sequenced genomes of serovar Typhi strains TY2 and CT18 (4, 29).

Adhesion of *E. coli* containing the *stg* operon. To examine the capacity of the *stg* fimbrial cluster to mediate adherence to INT-407 cells, the *stg* operon was cloned in different vectors and transformed into *E. coli* strain ORN172. ORN172 is an *E. coli* K-12 noninvasive strain with a deletion in the *fim* operon that does not express type 1 fimbriae and is commonly used to study adherence conferred by recombinant fimbrial systems (42).

E. coli ORN172 cells containing the vector alone (pCR2.1) adhered poorly between the cells or without pattern on the cell surface and were often isolated (Fig. 1A). However, ORN172 cells containing *stg* (pSIF018) adhered in aggregates or clusters on the cell surface (Fig. 1B). The introduction of *stg* into *E. coli* conferred a significantly higher level of adhesion to epithelial cells, which was threefold higher than that of the strain harboring the vector alone (Fig. 1C). A higher level of adhesion was also observed when a low-copy-number vector (pSIF026) was used (data not shown).

Adhesion of serovar Typhimurium containing the *stg* operon. As the *stg* fimbrial operon is absent in serovar Typhimurium, we used this serovar to establish whether *stg* could contribute to adherence to INT-407 cells by a heterogeneous *Salmonella* serovar. Serovar Typhimurium strain χ3339 harboring *stg* (pSIF018) exhibited a significantly higher level of adhesion to INT-407 cells, which was 30-fold higher than that of the strain harboring the vector alone (pCR2.1) (Fig. 1C). An invasion level similar to that exhibited by the wild-type parent harboring only the vector was observed (data not shown).

***stg* expression in serovar Typhi.** To study the expression of the *stg* fimbrial operon in the native serovar Typhi strain, an *stgA*::*lacZ* fusion was inserted into the chromosome of strain ISP1820 generating strain DEF068. Strain DEF068 was used to determine the influence of a number of in vitro growth conditions on *stg* expression. The expression of the promoter fusion was determined for bacteria grown in LB medium from early log phase to stationary phase. β-Galactosidase expression increased from early to stationary phase, following overnight growth in LB medium (Fig. 2). The β-galactosidase expression following growth on LB agar was nearly twofold higher (54 U) than the expression following overnight growth in LB broth (29 U) (Fig. 2). The highest levels of β-galactosidase expression were observed following overnight growth in minimal medium (M9-glucose) (76 U) (Fig. 2). Expression in conditions that mimic those encountered during invasion and infection of host cells was also studied. The effect of sodium chloride concentration in the medium was evaluated, as this concentration represents a condition that can influence cell invasion by *Salmonella* (1, 8). The effect of iron availability and pH on *stg* expression was also

evaluated. Changes in these conditions did not result in any significant changes in β-galactosidase expression (data not shown).

***stg* contributes to adherence of serovar Typhi to epithelial cells.** We assessed whether *stg* contributes to adherence of serovar Typhi to INT-407 cells by constructing an isogenic Δ*stgABCC'D* mutant by allelic exchange. The mutated strain, DEF004, exhibited a significantly lower level of adherence (80% of the wild-type strain adherence) (Fig. 3A). A level of adherence significantly higher than that of the wild-type strain was observed when the *stg* mutant was complemented with the *stg* genes on a low-copy-number vector (pSIF026) (Fig. 3A). In spite of the lower level of adherence of the mutant, its level of invasion was higher than that of the wild-type parent, but not significantly higher (Fig. 3A).

Loss of *stg* results in increased phagocytosis of serovar Typhi by macrophages. As survival in macrophages plays an essential role in systemic infection by *Salmonella*, we characterized the interaction of the isogenic *stg* mutant with human macrophages. The wild-type strain and the mutant were used to infect human macrophage-like cells, and the number of bacteria present after phagocytosis at 2 and 24 h postinfection were determined. The mutant showed a significantly higher level of phagocytosis than the wild-type strain (Fig. 3B). The levels of bacterial survival at 2 or 24 h postinfection were similar for both the *stg* mutant and the wild-type strain (Fig. 3B). Complementation of the *stg* mutant with *stg* on a low-copy-number vector (pSIF026) restored the wild-type phagocytosis phenotype (Fig. 3B).

Role of *stg* in macrophage interactions. As bacterial uptake of the *stg* mutant by macrophages was altered, we wanted to evaluate the effect of *stg* overexpression on phagocytosis. The uptake of both serovar Typhi strain ISP1820 and serovar Typhimurium strain χ3339 harboring *stg* (pSIF018) on a multicopy vector was significantly lower than the uptake of the bacterial strain harboring the vector alone (pCR2.1) (Fig. 4). This lower level of phagocytosis was also observed using macrophage-like U937 cells (data not shown). Then, in order to differentiate between the initial levels of bacteria associated with

or internalized by macrophages, we used an inhibitor of cytoskeletal function, cytochalasin D, to block bacterial uptake. In the presence of cytochalasin D, less then 2% of the initial inoculum was associated with macrophages. The percentages of serovar Typhi that were associated with macrophages were similar when *stg* was present at a high copy number and when the wild-type harboring the vector alone was used (Fig. 5). In addition, the *stg* mutant also showed a level of association with macrophages similar to that of the wild-type strain when bacterial uptake was inhibited by cytochalasin D (Fig. 5). Since the levels of association with macrophages were similar in cytochalasin D-treated cells regardless of the presence of *stg*, these results indicate that the *stg* fimbrial system contributes to a reduction in internalization of serovar Typhi by macrophages.

DISCUSSION

Bacterial adhesion to host cells is often considered an essential step for colonization. Adhesion is mediated via surface molecules, including fimbriae or pili. Many gene clusters corresponding to fimbrial systems are present in the genomes of *S. enterica*. However, only a few systems have been characterized, and only the *fim* cluster coding for type 1 fimbriae was detected after in vitro growth of serovar Typhimurium at 37°C in static broth (13). A combination of fimbrial systems may be responsible for the differences in binding and host specificities observed for different *Salmonella* serovars. Serovar Typhi is restricted to humans and harbors 13 putative fimbrial systems and a type IV pilus (29). We have previously found that *stg* is transcribed by serovar Typhi within macrophages (6). In *S. enterica*, the *stg* fimbrial cluster located in the *glmS-pstS* region is present only in serovar Typhi (30).

The *stg* gene cluster was suggested to be nonfunctional since the predicted ORF for the putative usher gene *stgC* contains an internal stop codon and is classified as a pseudogene (29, 39). Mutations in genes encoding assembly proteins, such as the usher, result in absence of fimbriae from the bacterial surface (18). The fimbrial usher protein family consists of a group of large proteins (800 to 900 aa) present in the outer membranes of gram-negative bacteria (40). The usher acts in the assembly process together with a periplasmic fimbrial chaperone protein. Phylogenetic analyses suggest that the chaperone and the usher, in general, evolved in parallel from their evolutionary precursor proteins (40). In bacteria expressing numerous fimbriae, each fimbrial system typically encodes a specific periplasmic chaperone protein and outer membrane usher protein (24, 34). However, fimbrial expression may be possible using complementary fimbrial proteins from other clusters. This is likely to occur with the LP fimbria-encoding *lpf1* cluster of *E. coli* O157:H7. This cluster contains a stop codon in the predicted usher-encoding gene which results in two ORFs, *lpf1C* (368 aa predicted) and *lpf1C'* (443 aa predicted) (37). The cloned *lpf1* gene cluster produced detectable fimbriae, and these fimbriae contributed to

microcolony formation, demonstrating that this system was therefore functional (37). The aims of our study were to characterize the *stg* fimbrial cluster and determine if this fimbrial cluster was functional despite the presence of a predicted pseudogene which comprises two ORFs, *stgC* (170 aa predicted) and *stgC'* (605 aa predicted), that may act as the usher. To circumvent the effect of the premature stop codon in the StgC usher gene, it is possible that other fimbrial ushers present in the cell may function for Stg; otherwise, the truncated StgC usher may be functional (24).

An increased level of association to epithelial cells was observed when the *stg* fimbrial cluster was cloned into a nonfimbriated *E. coli* strain (Fig. 1). We were unable to visualize any filamentous structures by transmission electron microscopy by negative staining. Other related fimbriae were also difficult to visualize and/or detect (26, 37, 38). Thus far, no studies have detected these fimbriae using wild-type strains, and fimbrial proteins or structures were detected only using an afimbrial recombinant *E. coli* strain and either multicopy or inducible vectors (21, 26, 37, 38). We were also unable to detect StgA when *stg* genes were cloned on a multicopy vector in *E. coli* or in *Salmonella* by Western blotting using an anti-StgA from *E. coli* (21). One explanation for the lack of fimbrial structures despite an adhesion phenotype may be that some export and partial assembly of the Stg protein occurs, which results in an adhesin that is not filamentous. Stg and related fimbriae exhibit a low level of transcription in vitro (26, 35, 37). This may also explain why these fimbriae are not readily detected in vitro. In serovar Typhi, using an *stgA-lacZ* single-copy fusion, a low level of *stg* expression was also detected in different growth conditions. The highest levels of *stg e*xpression were obtained when bacteria were grown in minimal medium or on solid medium (Fig. 2), and they were not influenced by the presence of salts or iron. The low level of fimbrial gene expression observed during in vitro growth of serovar Typhi is similar to results obtained with serovar Typhimurium (13). In serovar Typhimurium, which contains 13 fimbrial operons (22), only type 1 fimbriae were expressed in vitro at 37ºC. Similarly, the majority (11/15) of fimbrial clusters in *E. coli* O157:H7 were not expressed under the majority of conditions tested in vitro (20). It is currently not known why expression of many fimbrial systems is suppressed in vitro.

While they are an advantage to the bacterium for colonization of the host, fimbrial proteins at the bacterial surface may become a disadvantage, as they are easily exposed targets for the host immune system. Hence, tight regulation of fimbrial expression may be necessary during host infection. The induction of expression of fimbrial antigens during infection of mice with serovar Typhimurium was previously shown by seroconversion against most fimbriae (12). In typhoid fever patients, antibodies to 3 fimbrial systems, Tcf, Stb, and Csg, were detected (10). Nevertheless, we have previously detected the *stgC'* transcript during infection of macrophages (6). The optimal conditions for expression of Stg may not have been found yet, and we need to further investigate its regulation, but our results are consistent with the hypothesis that the *stg* fimbrial operon may be important for initial interaction with host cells.

When the *stg* operon was deleted from serovar Typhi, a lower level of bacterial association with INT-407 cells was observed (Fig. 3A). Further, a higher level of bacterial association with epithelial cells was observed when the *stg* mutant was complemented by the *stg* fimbrial cluster. In addition, an increased level of association with epithelial cells was observed when the *stg* gene cluster was introduced into *E. coli* and *S. enterica* serovar Typhimurium, in which *stg* is absent (Fig. 1C). These results implicate the *stg* fimbrial operon in host cell interaction. The stg operon and the type IV pili are the only serovar Typhi determinants identified so far that confer adherence to human epithelial cells (43). Redundancy of virulence determinants is not uncommon. Wild-type virulent serovar Typhi strains lacking SPI-7, which harbors type IV pili, have been isolated (25), suggesting that the *stg* fimbrial operon may confer adhesion to host cells in Δ*pil* strains. The *stg* fimbrial cluster may represent an additional system for host intestinal colonization. Many functions have been associated with fimbriae related to Stg. In avian pathogenic *E. coli*, Stg contributes to the colonization of avian respiratory tissues (21). In *E. coli* O157:H7, long-term persistence in sheep and pigs was associated with the presence of Lpf1 and Lpf2 (14), which also influenced intestinal tissue tropism (7). In rabbit enteropathogenic *E. coli*, Lpf_{R141} is involved in initial colonization (26).

Although loss of *stg* genes reduced the adherence of serovar Typhi to epithelial cells, a higher level of phagocytosis was observed with the *stg* mutant (Fig. 3B). Further, a lower level of phagocytosis was observed when *stg* was overexpressed in serovar Typhi, as well as in serovar Typhimurium (Fig. 4). The higher level of phagocytosis in the absence of the *stg* genes may have been caused by increased exposure of different bacterial surface proteins that are more readily recognized by macrophages, thus enhancing macrophage association. To rule out this possibility, bacterial association with macrophages was assessed in the presence of cytochalasin D, an inhibitor of actin polymerization, which mediates uptake of bacteria. The numbers of bacteria associated with cytochalasin D-treated macrophages were similar for the wild-type, the *stg* mutant strain, and a strain overexpressing *stg* (Fig. 5). Thus, the higher level of phagocytosis observed with the mutant was not the result of increased exposure of other proteins on the bacterial surface that may have increased association with phagocytes. Similarly, the lower level of phagocytosis observed when the *stg* fimbrial cluster was overexpressed was not due to a decrease in the association with macrophages but was likely due to a specific reduction in phagocytic activity. By contrast, type IV pili increased entry of serovar Typhi in macrophages (28). This suggests that Stg and type IV pili use different interaction mechanisms with host cells. The level of invasion of INT-407 cells and intracellular survival in human macrophages of strains with *stg* or the mutant were similar to the results for the wild-type strain even when bacterial uptake by macrophages was inhibited (Fig. 3, 5). This favors the hypothesis that the presence of the *stg* genes may be involved primarily in initial contact with host cells. It is possible that the *stg* fimbrial operon may promote inhibition of phagocytosis in order to evade inflammatory cells of the intestine so that the bacteria can invade deeper tissue.

The data presented in this paper demonstrate that the *stg* gene cluster of serovar Typhi expresses a functional and serovar-specific adhesin. The *stg* gene cluster potentially contributes to the initial stages of typhoid fever pathogenesis by mediating adherence of serovar Typhi to host epithelial cells and by inhibiting phagocytosis. It is important to understand this inhibition mechanism, to characterize the regulation, expression, and

production of Stg in vivo, and determine if Stg possesses a specific host cell receptor that may be a potential target for the prevention of typhoid fever.

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TABLES AND FIGURES

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristic(s)	Source or reference
S. enterica serovar Typhi strains		
ISP1820	Wild type	R Curtiss III (Arizona State University)
DEF004	ISP1820 Δ*stg*	This study
DEF033	ISP1820 (pSIF018)	This study
DEF064	ISP1820 (pCR2.1)	This study
DEF066	DEF004 (pSIF026)	This study
DEF068	ISP1820 :: P*stgA-lacZ* (::pSIF020)	This study
S. enterica serovar Typhimurium strains		
χ3339	Mouse-passaged isolate of SL1344 *rpsL hisG*	9
DEF047	χ3339 (pSIF018)	This study
DEF048	χ3339 (pCR2.1)	This study
E. coli strains		
DEF045	ORN172 (pCR2.1)	This study
DEF049	ORN172 (pSIF018)	This study
MGN-617	SM10 λpir *asd thi thr leu tonA lacY supE recA* RP4 2-Tc ::Mu[λpir] Δ*asdA4*	15
ORN172	*thr-l leuB thi-1 Δ(argF-lac)U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44* Δ(*fimBEACDFGH*)::*kan pilG1*	42
Plasmids		
pCR2.1	High-copy-number cloning vector, Km^r Ap^r	Invitrogen
pFUSE	*lacZYA mob*$^+$ (RP4), R6K *ori* (suicide vector), Cm^r	2
pMEG-375	*sacRB mobRP4 ori*R6K. Cm^r Ap^r	Megan Health (St. Louis, MO)
pSIF004	Suicide vector with flanking region of *stgA* in 5' end and *stgD* in 3' end used for *stg* deletion	This study
pSIF016	pCR2.1 carrying a 530-pb fragment of *stgA*	This study
pSIF018	pCR2.1 carrying a 5-kb fragment of *stg* (pCR2.1*stg)*	This study
pSIF020	pFUSE carrying a 530-pb fragment of *stgA*, Cm^r	This study
pSIF026	pWSK29 carrying a 5-kb fragment of *stg* (pWSK*stg*)	This study
pWSK29	Low-copy-number cloning vector, Amp^r	41

Table 2. Comparison of the *stg* fimbrial gene products of *Salmonella* serovar Typhi with other fimbrial systems[a]

Fimbrial group	Organism	Localization	% Identity (% similarity)			
			StgA	StgB	StgC[b]	StgD
Stg	*S. bongori*	*glmS-pstS*	70.7 (81.2)	63.7 (73,7)	82.2 (89.8)	38.7 (53.6)
	Avian pathogenic *E. coli* 078 (*stg*)	*glmS-pstS*	66.5 (79.1)	54.8 (69.8)	67.1 (82.0)	36.6 (54.1)
	Enterohemorrhagic *E. coli* O157 (*lpf2*)	*glmS-pstS*	59.4 (72.8)	62.1 [b] (76.3)	73.5 (85.6)	35.4 (50.3)
	Enterohemorrhagic *E. coli* O113 (lpf_{O113})	*glmS-pstS*	66.5 (79.1)	53.6 (69.4)	67.1 (82.0)	24.7 (37.9)
Lpf	*S. enterica* serovar Typhimurium LT2	*yhjX-yhjW*	32.5 (45.7)	32.1 (53.3)	40.8 (59.2)	27.9 (45.6)
	Enterohemorrhagic *E. coli* O157 (*lpf1*)	*yhjX-yhjW*	30.5 (44.2)	33.9 (54.8)	38.8 [b] (56.6)	28.0 (44.6)
	Rabbit enteropathogenic *E. coli* O15	*yhjX-yhjW*	35.2 (48.2)	30.0 (52.2)	41.1 (58.4)	27.3 (42.2)

[a] Sequences were obtained from coliBASE (http://colibase.bham.ac.uk/).

[b] A complete ORF was used for comparison analysis.

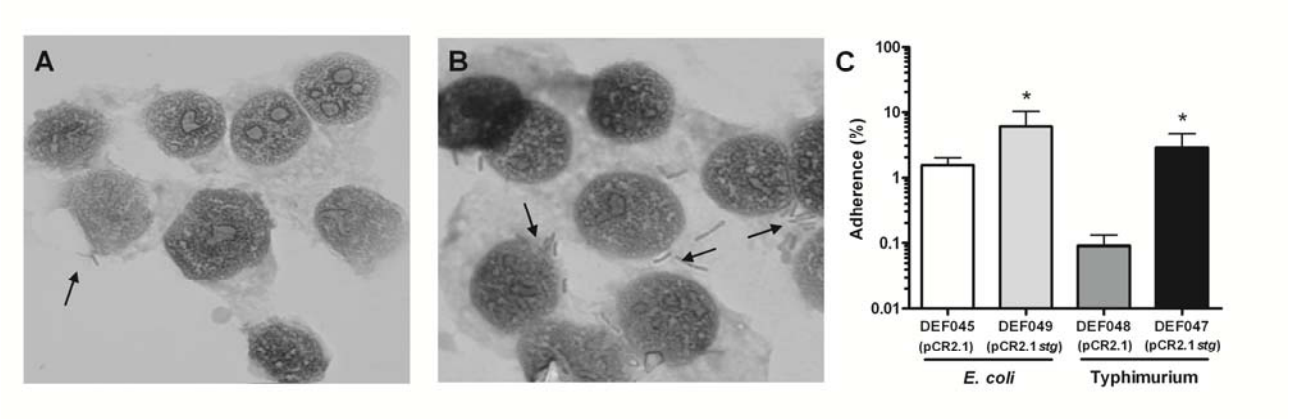

Figure 1. Adherence and expression of the *stg* fimbrial operon by *E. coli* and *S. enterica* serovar Typhimurium. (A and B) Adherence of *E. coli* strain ORN172 to human epithelial cells (INT-407) containing the vector (pCR2.1) (DEF045) (A) or the *stg* genes (pSIF018) (DEF049) (B). Slides were stained with 5% Giemsa stain. Bacteria are indicated by arrows. (C) Percentage of the initial inoculum associated with epithelial cells after 90 min of incubation for *E. coli* and serovar Typhimurium carrying the *stg* operon (DEF047) or the control vector (DEF048). All assays were conducted in duplicate and repeated independently at least three times. The results are expressed as the mean ± standard error of the replicate experiments. An asterisk indicates that there is a difference between the strain containing the control vector and the strain containing the *stg* operon ($P < 0.005$).

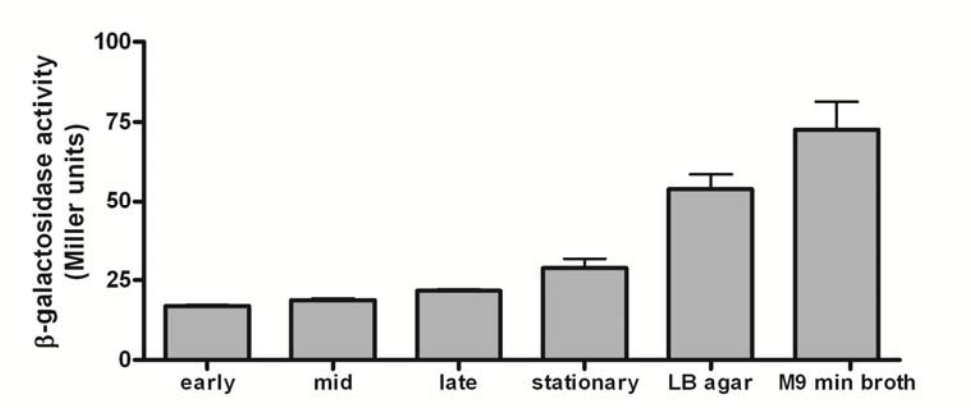

Figure 2. *stg* expression in serovar Typhi: β-galactosidase activity expressed from the P*stgA*::*lacZ* fusion in serovar Typhi (DEF068) in different growth conditions. Bacteria were grown in LB medium with agitation to early log phase (OD_{600}, 0.3), mid-log phase (OD_{600}, 0.6), late log phase (OD_{600}, 0.9), and stationary phase (overnight), on LB agar and in M9-glucose broth (M9 min broth) (overnight). The error bars indicate standard deviations.

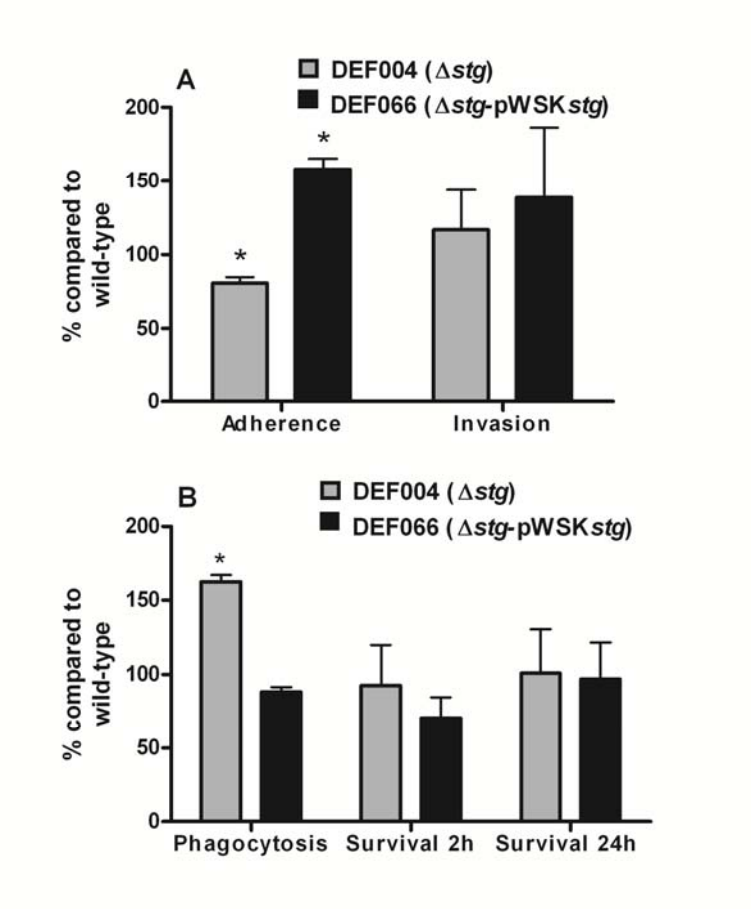

Figure 3. Role of *stg* in the interaction of serovar Typhi with human cells: capacity of the wild-type strain, the *stg* mutant (DEF004), and the complemented strain (DEF066) to adhere to and invade INT-407 cells (A) or to survive within THP-1 macrophage-like cells (B). All assays were conducted in duplicate and repeated independently at least three times. The results are expressed as the means ± standard errors of the replicate experiments. Significant differences ($P < 0.005$) in adherence or phagocytosis between the mutant and the wild-type strain of serovar Typhi are indicated by asterisks. The values for percent recovery were normalized to the wild-type control value, which was defined as 100% at each time point.

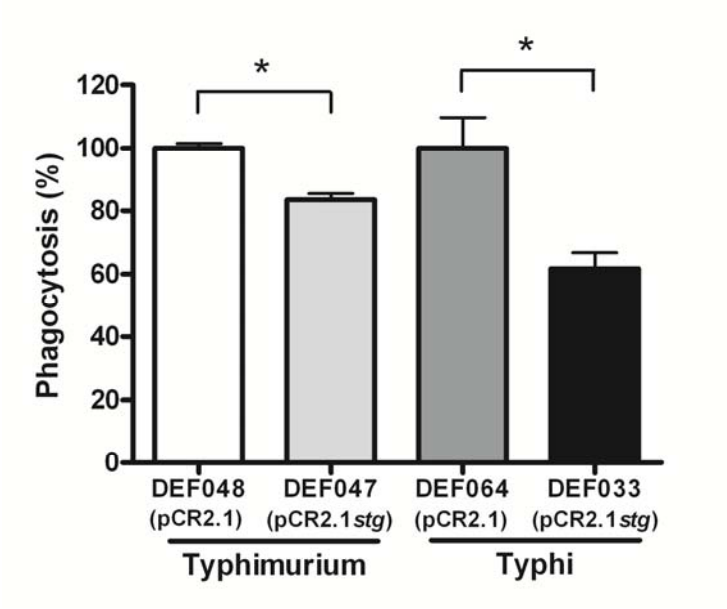

Figure 4. Effect of overexpression of *stg* on phagocytosis. Serovar Typhimurium carrying the *stg* cluster (DEF047) or the control vector (DEF048) and serovar Typhi carrying the *stg* cluster (DEF033) or the control vector (DEF064) were incubated with THP-1 macrophage-like cells. The percentage of the initial inoculum associated with cells after 120 min of incubation is indicated. All assays were conducted in duplicate and repeated independently at least three times. The results are expressed as the means ± standard errors of the replicate experiments. An asterisk indicates that there is a significant difference in phagocytosis between the wild-type strain containing the vector alone and the strain with the *stg* operon ($P < 0.05$).

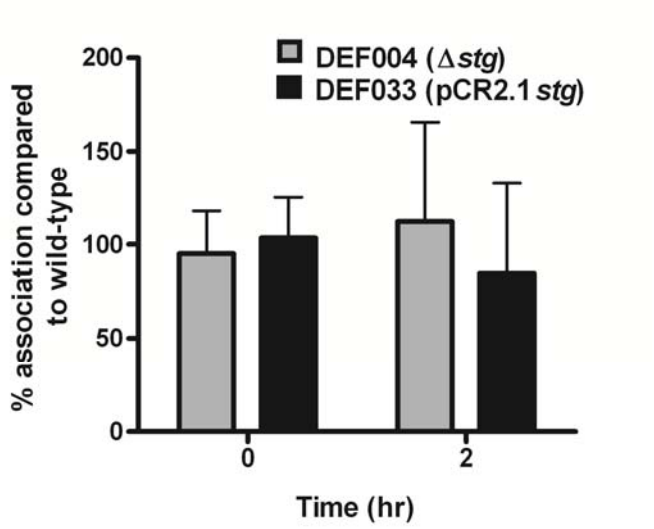

Figure 5. Role of *stg* fimbrial operon in bacterial association with macrophages. Bacterial uptake was inhibited with cytochalasin D, and the numbers of bacteria with *stg* (DEF033) and without *stg* (DEF004) associated with macrophages were compared. All assays were conducted in duplicate and repeated independently at least three times. The values for percent recovery were normalized to the wild-type control value, which was defined as 100% at each time point. The results are expressed as the means ± standard errors of the replicate experiments.

Préface au Chapitre 3

La seconde étape de ce projet consistait à déterminer le mécanisme moléculaire permettant l'expression à la surface des sous-unités fimbriaires de l'opéron *stg*. Différentes mutations ont été effectuées dans *stgABCD* et l'exportation de l'adhésine StgD a été évaluée par Western blot et par microscopie électronique à transmission. La traduction du pseudogène *stgC* a été étudiée par l'insertion d'une étiquette 6-histidines en C-terminal. Une fusion traductionnelle entre *stgC* et la *gfp* a permis d'évaluer le taux de suppression du codon d'arrêt TAA prématuré. Cet article sera soumis à un processus de révision par les pairs prochainement dans le but de le publier dans le journal « Nucleic Acids Research ».

Article 3 : **Forest, C.G.**, Houle, S., Dozois, C. M. and Daigle, F. Recoding of a TAA stop codon of a bacterial pseudogene leads to production of Stg fimbriae.

Contribution des auteurs :

Le projet a été élaboré par France Daigle et moi-même. J'ai réalisé toutes les expérimentations relatives au projet (création d'amorces, souches et expériences). La microscopie électronique a été réalisée avec l'aide de Sébastien Houle et Charles M. Dozois. En tant que première auteure, j'ai contribué de façon majoritaire à l'analyse des résultats et à l'écriture du manuscrit, avec les commentaires de France Daigle et de Charles M. Dozois. Tous les coauteurs ont révisé le manuscrit. Les fonds nécessaires au projet ont été fournis par France Daigle.

CHAPITRE 3, 3e article

Recoding of a TAA stop codon of a bacterial pseudogene leads to production of Stg fimbriae

Running title : Recoding of a TAA stop codon in bacteria

Chantal G. Forest, Sébastien Houle, Charles M. Dozois and France Daigle

Keywords: pseudogene; recoding; stop codon; readthrough; *Salmonella*; fimbriae

ABSTRACT

The bacterial genome of *Salmonella enterica* serovar Typhi features a high prevalence of pseudogenes, such as *stgC* that harbors a premature TAA stop codon. Recoding of stop codons is rare in bacteria, hence pseudogenes are normally considered non-functional. However, previous results showed that the *stg* fimbrial system plays a role during interaction with host cells. As *stgC* corresponds to a predicted usher required for export and specific assembly of fimbrial subunits at the cell surface, translocation of fimbrial subunits was evaluated. The fimbrial subunits were detected in cell surface extracts of *Escherichia coli* containing the *stg* gene cluster and thin fibrillae were visualized, indicating active export and assembly by the *stgC* pseudogene product. Moreover, a 90 kDa protein, corresponding to the full size of the predicted usher without a premature stop codon, was produced by *stgC*. StgC sequencing revealed that a tyrosine was inserted at the stop codon, suggesting a readthrough mechanism. Monitoring efficiency of translation in single cells of *S*. Typhi revealed 0.8% readthrough. This is the first experimental demonstration of a natural TAA stop codon recoding in bacteria. Hence, pseudogenes may in some cases be functional, and recoding may represent a novel regulatory mechanism.

INTRODUCTION

Evolution of bacterial pathogens occurs through gene acquisition and genome degradation. Insertions or mutations such as nonsense substitutions, frameshifts, rearrangement or partial deletion lead to loss of gene function. Alteration of more than 20% of the amino acid sequence of a predicted open reading frame generates a pseudogene, which is then considered to be non-functional (1). There is no strict consensus regarding the presence of pseudogenes in bacterial genomes. Some argue that pseudogene accumulation is a direct consequence of a loss of gene function based on the fact that intracellular and host-specific bacteria harbor more pseudogenes than free-living organisms (2-6). However, much evidence points to the possibility that pseudogenes can still be transcribed, translated or functional (7). First, significant changes in transcriptional profiles are often observed for pseudogenes during transcriptional studies (8-12). More strikingly, some proteomic studies show clear evidence of pseudogene translation (11) and antibodies directed against pseudogenes products have been detected in host sera (13-15). Despite these facts, there is a lack of research concerning the biological relevance of the presence of pseudogenes in bacteria.

Salmonella enterica serovar Typhi (*S.* Typhi) is a monomorphic bacterium, showing very little genetic diversity (16), is restricted to humans and up to 5% of its annotated coding sequences are pseudogenes (17,18). Among the 204 pseudogenes present in *S*. Typhi strain CT18, 51 harbor premature stop codons, mostly TAG (57%), then TGA (30%) and finally TAA (13%) (Table S1). Suppression of an ochre stop codon (TAA) in bacteria is a very rare event and TAA is considered to be the most efficient termination signal since both release factors (RF1 and RF2) are active at this site (19). *stgC* (STY3920) harbors a premature TAA stop codon at the 171st codon, that is conserved in all the sequenced strains of *S*. Typhi (20). The *stgC* pseudogene is part of the *stgABCD* operon containing genes predicted to encode filamentous surface structures called fimbriae mainly involved in adhesion. The *stg* operon was initially considered non-functional (21,22). However, previous studies have shown that certain fimbrial operons harboring a disrupted usher can

still produce fimbriae at the bacterial surface, but the mechanisms underlying fimbrial biogenesis remain unknown (23-25). Moreover, deletion of *stg* genes in a strain of *S.* Typhi caused a decrease in adhesion to epithelial cells and enhanced phagocytosis of this strain by macrophages, suggesting that the Stg fimbrial system is functional (26). Furthermore, *stgC* transcription was observed during infection of macrophages (8,27). Stg fimbrial biogenesis occurs through the chaperone/usher pathway. This mechanism normally involves at least four different proteins: a major subunit (StgA), a minor subunit (StgD), a chaperone (StgB) and an outer membrane (OM) usher (StgC). For efficient translocation to the bacterial surface, the subunits are first secreted by the Sec general secretory pathway in the periplasm (28) where they interact with the chaperone. The specific interaction between the chaperone and fimbrial subunits ensures their correct folding, prevents an early assembly with other subunits and protects them from degradation (29-31). The chaperone-subunit complexes interact with the usher, a β-barrel anchored in the OM. The usher constitutes the channel used for subunit translocation and catalyses fimbrial assembly (32-34). When the usher is deleted from a fimbrial operon, complexes remain in the periplasm and are not translocated to the bacterial cell surface (32,35,36).

Some hypotheses may explain how a fimbrial operon harboring a truncated usher can translocate subunits on the bacterial surface. Complementation by an usher from another fimbrial system may occur, as previously seen for FocD/FimD and PapC/FimD ushers in *E. coli* (37,38). Alternatively, a recoding mechanism allowing stop codon readthrough such a tRNA suppressor or translational hopping could lead to translation of StgC (39). In this study, we investigated the role of the *stgC* pseudogene for the Stg fimbrial subunit translocation and assembly. Our results showed that the *stgC* pseudogene can encode a functional usher that is essential for the translocation of Stg fimbrial subunits by a mechanism that involves TAA stop codon readthrough.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are described in Table S2. Bacteria were routinely grown overnight with agitation in Luria-Bertani (LB) Miller broth at 37°C, except when indicated. When required, antibiotics were added at the following concentrations: kanamycin (50 μg/ml), ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). Transformation of *E. coli* strains was done by using the calcium/manganese based (CCMB) method (40) and electroporation of *Salmonella* was done as described (41).

Generation of mutations in the *stgABCD* fimbrial system. Mutations were generated in the *stgABCD* fimbrial operon (Fig. 1A, Tables S2 and S3) by the PCR overlapping method as described (42). Genomic DNA of *Salmonella enterica* serovar Typhi strain ISP1820 was used as DNA template and amplified with the Elongase® enzyme mix (Invitrogen). Site-directed mutagenesis of the TAA stop codon to a TAT codon (tyrosine) in *stgC* was achieved using the same method. The resulting constructs as well as the wild-type *stgABCD* operon were digested with *Eco*RI and *Xba*I restriction enzymes and inserted in the pBAD24 arabinose-inducible vector. All mutations were confirmed by PCR sequencing and transformed into afimbriated *E. coli* strain ORN172. For complementation of the *stgC* deletion, the *stgC* pseudogene was cloned in pBAD33 at the *Xba*I and *Hin*dIII restriction sites. The stop codon in *stgC* was very stable as confirmed by three independent sequencing verifications of the strain harboring the wild-type version of the *stgABCD* fimbrial system during the experiments.

Protein purification and generation of antibodies. The *stgD* gene of *S.* Typhi was amplified with Platinum® *Taq* DNA Polymerase enzyme mix (Invitrogen) using primers stgDF-*Nde*I no SS and stgDR-*Xho*I to generate a mature StgD lacking the signal sequence. The leader peptide signal sequence was removed in order to purify proteins from the inclusion bodies. After a double digest, a 999 bp fragment was ligated into pET-21a vector (C-terminal hexahistidine tag, Novagen) and transformed into *E. coli* BL21 (DE3) cells.

After 3 hours of protein induction in LB broth supplemented with 1 mM isopropyl-β-D-thiogalactoside (IPTG), cells were sonicated and StgD proteins were purified from inclusion bodies (43). StgD was further purified using the HiTrap system (Amersham) and purified proteins were sent for rabbit polyclonal antibody production (Animal Resources Centre, McGill University). Antiserum was absorbed with whole cell lysate of a Δ*stgD* strain (DEF189) induced for 1 hour with 0.05% L-arabinose and grown at 37°C.

Analysis of subunit translocation. Bacteria were grown overnight on tryptic soy agar plates supplemented with 0.05% arabinose for fimbrial induction (0.2% for the complemented strain DEF489). Cells were harvested from plates with LB broth, resuspended by vortexing and cell surface proteins were extracted at 60°C for 15 minutes. Bacteria were pelleted by centrifugation (3 000 *g* for 10 min.) and the supernatant was precipitated with 10% trichloroacetic acid as previously described (44). The amount of proteins was normalized according to the OD_{600} value of each bacterial suspension. Proteins were loaded in a 12% SDS-PAGE gel, transferred to a PVDF membrane following migration and a Western blot was performed with anti-StgD serum as previously described (44). The experiment was done with three different protein extractions.

Visualization of fimbrial production. Induced cultures were recovered by centrifugation and allowed to adhere to a carbon-Formvar-coated copper grid. After 1 minute of negative staining with phosphotungstate 1%, grids were allowed to dry and analysed with a Phillips EM300 electron microscope.

StgC usher production and sequencing. The *stgC* usher was amplified from genomic DNA of *S.* Typhi using Elongase® enzyme mix (Invitrogen) with the primers stgCF-*Nde*I and stgC'R 21 to generate a mature StgC lacking the signal sequence. The 2460 bp PCR product was purified and cloned into vector pET21a resulting in plasmid pSIF110. The $stgC^{TAT}$ fragment, harboring the usher sequence without the premature stop codon (TAA→TAT), was amplified using pSIF067 as the template DNA and cloned in pET21a resulting in plasmid pSIF111. The exact sequences of the clones were confirmed by

sequencing. A shorter version of *stgC*$^{42\ kDa}$ was amplified from genomic DNA of *S.* Typhi using stgCF-*Nde*I and stgC40R primers and cloned in pET21a resulting in plasmid pSIF169. The primers were generated to yield a protein of about 42 kDa after induction. The different constructs were transformed into *E. coli* BL21 (DE3) cells.

For usher induction, overnight cultures grown in tryptic soy broth at 37°C were diluted 1/50 in 500 ml of tryptic soy broth without glucose and grown to an OD_{600} value of 0.5. The induction was done for 2 hours in the presence of 1 mM IPTG and proteins were purified from inclusion bodies (45). For protein sequencing analysis, the usher was further purified using Ni-nitrilotriacetic acid (NTA) agarose beads (QIAGEN) and concentrated by using Amicon Ultra centrifugal filter devices (Millipore). After a trypsin digestion, peptides were sequenced using LC-MS/MS.

Construction of translational fusions with *gfp*. Codons 151 to 181 surrounding the premature stop codon of *stgC* were fused to *gfp* in pXG-10 (46). Constitutive transcription was achieved from a P_{LtetO} promoter. The +1 site of initiation of transcription, the RBS and ATG codon of pBAD18s were inserted upstream of the thirty codons from *stgC*. The *stgC*^{TAA} pseudogene sequence, the *stgC*^{TAT} allele or a *stgC* sequence harboring 2 stop codons (*stgC*^{TAATGA}) was amplified with Taq DNA Polymerase (Feldan Bio) using stgCF *Nsi*I and stgCR *Nhe*I primers. The amplicons were digested with *Nsi*I and *Nhe*I restriction enzymes and inserted in the pXG-10 vector (46). All the mutations were confirmed by PCR. Purified plasmids were electroporated in *S.* Typhi strain ISP1820 and all the mutations in *stgC* were reconfirmed by PCR sequencing.

Quantification of TAA stop codon readthrough in *S.* Typhi. To measure single-cell fluorescence in liquid culture by flow cytometry, strains harboring translational GFP fusion plasmids were grown overnight in LB broth with aeration at 37°C and resuspended in PBS (pH 7.4). Cultures were fixed with 2% paraformaldehyde prior to each analysis. *stgC*^{TAT} was used as positive control for GFP expression and *stgC*^{TAATGA} was used as a negative control. The data from 50 000 events acquired on a BD FACSort were analyzed with the

CellQuest program. To calculate relative fluorescence for stop codon readthrough, data were normalised by substracting the value obtained for the corresponding negative control and then were divided by the fluorescence of the corresponding positive control. The experiment was repeated at least three distinct times.

RESULTS

Translocation of fimbrial subunits. Fimbrial ushers translocate and assemble subunits at the bacterial cell surface. To establish the role and impact of the premature TAA codon in the StgC usher on fimbrial production and assembly, different genetic modifications of the *stgABCD* system were generated (Fig. 1A). Subunit translocation was measured using polyclonal anti-StgD serum. Analysis of cell surface extracts obtained by heat shock to detach bacterial surface proteins revealed a specific band of 35 kDa in cell surface extracts and in the whole cell proteins of the strain harboring the complete wild-type *stgABCD* fimbrial genes (Fig. 1B). This band was completely absent in cell surface extracts and in the whole cell fraction of a strain harboring a complete deletion in *stgD*, confirming the specificity of the anti-StgD serum. StgD proteins were detected in the extracts of the strain lacking the major subunit *stgA*, but some degradation of StgD was also observed. The strain containing a plasmid lacking *stgC* (Δ*stgC*) was unable to efficiently assemble StgD on the surface of bacteria, which was produced in the whole cell fraction (Fig. 1B). Introduction of *stgC* on a second plasmid did not complemented the strain probably due to the fact that transcription of the genes present in a fimbrial operon must be transcribed with a specific stoichiometry (47,48). The substitution of the premature stop codon to a tyrosine codon (TAA to TAT) in *stgC* also resulted in production in the whole cell fraction and translocation of StgD at the bacterial surface (Fig. 1B). Thus, this experiment confirmed our hypothesis that translocation of Stg subunits specifically required the presence of the StgC usher.

Stg fimbriae visualised by electron microscopy. In order to visualise the structures produced by the *stg* fimbrial system, electron microscopy was performed on *E. coli* ORN172 harboring the *stg* operon containing either the wild-type $stgC^{TAA}$ gene, the $stgC^{TAT}$ allele or lacking *stgC* (Δ*stgC*). When $stgC^{TAA}$ or $stgC^{TAT}$ were present, thin flexible fibrillar appendages of about 3 to 4 nm in width were observed (Figs. 2B-C). These structures correspond to Stg since no fimbria was observed in the absence of the *stgC* usher (Fig. 2A) and fimbrial structures were not detected from cell surface extracts (Fig. 1B). By contrast,

when the *stgC*^{TAT} or the *stgC*^{TAA} wild-type usher were present, fimbrial structures were observed associated with bacterial cells (Fig. 2C) or detached from the bacterial cell surface (Fig. 2B) respectively. Thus, presence of fimbrial structures confirmed an efficient assembly of fimbrial subunits by the StgC usher.

Production of the StgC fimbrial usher. In order to understand how the Stg fimbrial subunits are translocated, the production of the pseudogene StgC usher was investigated using a 6-Histidine tag introduced at its C-terminal end (Fig. 3A). The usher sequence without premature stop codon, StgC^{TAT}, was used as a positive control for production of the full ORF. Analyses of inclusion bodies of induced cultures by Coomassie-stained SDS-PAGE revealed an abundant polypeptide at ~ 16 kDa, suggesting that most of the *stgC* translation stopped at the 171st premature TAA stop codon. When the stop codon was replaced (StgC^{TAT}), a strong band was observed at about 90 kDa corresponding to the full-length StgC product (Fig. 3B) and confirmed by Western blot using anti-6His antibodies (Fig. 3C). Unexpectedly, the *stgC* pseudogene also produced a small amount of the full-length 90 kDa protein (Fig. 3C). Thus, the predicted StgC usher is produced despite the presence of a premature stop codon.

StgC amino acid sequencing. To understand the event allowing full translation of the pseudogene *stgC* usher, purification of the 90 kDa proteins was attempted, but the amount of recovered proteins was too low for sequencing. A shorter version of *stgC*^{TAA} was cloned to obtain a protein of 42 kDa in order to enhance protein recovery (Fig. 4A). A significant amount of the 42 kDa protein was produced and purified (Fig. 4B) although most of the translation ceased at the stop codon (Fig. 4B). The presence of the predicted StgC truncated version of 42 kDa was revealed by Western blot in addition to three other bands of lower molecular weight (Fig. 4C). The four bands were extracted separately and sent for amino acid sequencing. The insertion of a tyrosine at the stop codon in the 42 kDa truncated StgC was identified in two different purifications (Fig. 4D). The lower molecular weight bands also corresponded to the predicted amino acid sequence of StgC. No frameshifting event was observed since an alignment of the amino acids obtained after sequencing

demonstrated that sequences 3' to the stop codon corresponded to the expected *stgC* encoded product (Fig. 4D). Thus, StgC is produced by a mechanism that allowed readthrough of the TAA stop codon.

Quantification of TAA readthrough at the single-cell level in *S.* Typhi. A translational fusion between the region surrounding *stgC* stop codon and *gfp* was made to quantify the TAA stop codon readthrough at the single-cell level (Fig. 5A). The TAA stop codon modified for a TAT codon was used as positive control, while 2 consecutive stop codons (TAATGA) were used as negative control for GFP expression. Under the tested conditions, about 0.8% of the cells demonstrated readthrough and translated GFP in *S.* Typhi as quantified using flow cytometry (Fig. 5B). This recoding event was confirmed by immunoblot performed with anti-GFP serum (data not shown).

RNA secondary structure prediction. The conformational structure surrounding the stop codon was evaluated using the Mfold program (49). Interestingly, a stem-loop structure was predicted immediately after the stop codon (Fig. 5C).

DISCUSSION

Over 5% of the predicted ORFs in the *S.* Typhi genome are pseudogenes (17). Although it is strongly believed that pseudogenes are inactive and non functional in bacteria and constitute "junk DNA" (50), deletion of the *stg* operon containing a pseudogene usher resulted in decreased adherence to epithelial cells and increased phagocytosis levels (26). In this study, the role played by the *stgC* pseudogene for translocation of StgD fimbrial subunit to the cell surface was assessed. Interestingly, deletion of the *stgC* pseudogene abrogated surface localisation of StgD, although StgD was detected in whole cell lysates (Fig. 1B). As translocation of StgD subunits to the cell surface requires StgC and no complementation by other cryptic fimbrial ushers occurred, StgC production from the pseudogene was sufficient for fimbrial biogenesis. Finally, electron microscopy showed the production of thin fimbriae when the *stgC* pseudogene or the modified $stgC^{TAT}$ gene products were present (Fig. 2). Detection of long thin rods provides further evidence that the predicted major fimbrial subunit StgA, as well as the pilus tip StgD, are efficiently translocated by the StgC usher in order to produce fibrillar appendages. Besides type 1 fimbriae, this is the only other fimbriae of *S.* Typhi expressed by the chaperone/usher pathway to be visualised so far (51).

During the production of StgC, most of the translation seemed to stop at the premature TAA stop codon, as a highly abundant 16 kDa band was observed (Fig. 3B), but the detection of a full-length 90 kDa suggested that some stop codon readthrough lead to the translation of the StgC usher (Fig. 3C). Stop codon readthrough can be observed in all organisms and in some cases, the universal code was naturally modified to insert amino acids at a stop codon (see (52) for a complete review). Many viruses actively use recoding events like frameshifting or stop codon readthrough as regulatory mechanisms, but few relevant examples are actually known for bacteria (see the RECODE database for a complete list of translational recoding events at http://recode.ucc.ie/ (53)). Insertion of selenocysteine at the TGA stop codon is the programmed readthrough event mostly observed and studied in prokaryotes (54). Interestingly, in eubacteria, the only other known

biological example of readthrough of a stop codon involved the synthesis of CS3 pili of the enterotoxigenic *E. coli* (55). In this case, the recoding event incorporated glutamine at a UAG stop codon by a suppressor tRNA. Recoding by a tRNA suppressor requires at least two copies of the same tRNA. Since *S.* Typhi possesses only one copy of $tRNA^{tyr}$ (GUA anticodon), the best hypothesis explaining *stgC* recoding would be an A-G mismatch at the third position of the codon. According to the rules proposed by Lim and Curran (56) based on analyses of the ribosome decoding center, a G-A base pairing at the third codon position can promote misreading.

Recoding events are scarce in bacteria and difficult to predict, as there is no consensus motif or structure (57). Secondary structures in the mRNA as well as the surrounding sequences can promote a recoding event (58). A stem-loop structure located directly downstream of the TAA stop codon might slow the ribosome or affect its efficiency and promote recoding in *stgC* (Fig. 4C). The role played by this structure will need to be investigated by modifying the sequence in order to destabilise this hairpin structure. The efficiency of translation termination is also sensitive to sequences upstream and downstream of the stop signal (59,60). The preferred codons in 5' of a TAA stop codon for termination in *E. coli* were not observed in *stgC* (61), while in 3', the termination efficiency of TAA followed by a G, as observed in *stgC*, was only 50% (62), supporting occurrence of readthrough events.

The majority of the previous studies on stop codon readthrough originated with the "synthetic" incorporation of a stop codon in reporter genes like β-galactosidase, luciferase, green fluorescent protein and protein A of *S. aureus* (7,63,64). Glutamine (CAA) was the only amino acid reported to be incorporated at a TAA stop codon in a suppressor-free strain of *E. coli* (64). Our study showed for the first time the functionality of a natural leaky stop codon, in this case, by measuring exportation of fimbrial subunits.

A recent elegant study demonstrated that in *Bacillus subtilis*, the translational machinery is error prone mostly in stressful conditions and may contribute to enhance bacterial fitness

(65). For example, cells harboring a stop codon in the chloramphenicol resistance gene can translate a pool of heterogeneous proteins from the same transcript and develop antibiotic resistance (65). In our study, the stop codon bypass was evaluated at 0.8% in *S.* Typhi when compared to the *stgC*^{TAT} allele. The presence of a leaky stop codon in *stgC* might be a translational mechanism to downregulate Stg fimbrial expression. Indeed, if only a small amount of the full-length StgC usher is translated, consequently this will limit the overall surface expression of StgD and StgA subunits. Fimbriae are surface-exposed and easily recognisable by the immune system, hence bacterial pathogens possess many mechanisms to limit their expression only when absolutely required (66). Stop codon readthrough might be a novel mechanism used to regulate expression of bacterial determinant. Since *S.* Typhi harbors five putative fimbrial systems with at least one pseudogene ORF, as well as many other pseudogenes, further efforts should be undertaken to evaluate their possible functionality.

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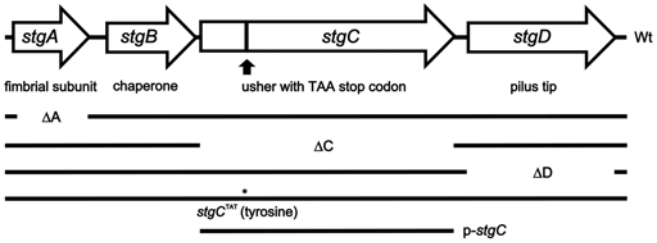
FIGURES

A)



B)

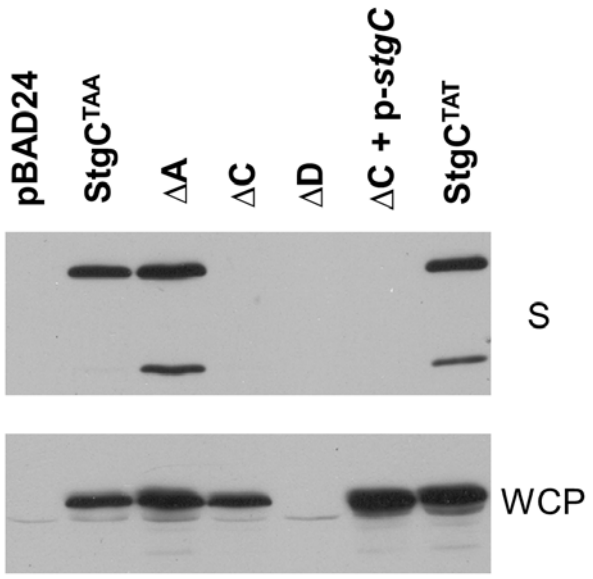


Figure 1. Mutations generated in the *stgABCD* fimbrial genes and translocation of StgD subunits at the cell surface. (A) Illustration of the different *stgABCD* fimbrial gene constructs cloned into the pBAD24 vector. Wt indicates $stgC^{TAA}$ pseudogene, the asterisk shows the site-directed mutagenesis ($stgC^{TAT}$) and p-*stgC* represents pBAD33*stgC* complementation plasmid. (B) Immunoblot of cell surface extracts treated with anti-StgD polyclonal antibody after arabinose induction in ORN172 harboring pBAD24 or the different modifications inserted in *stgABCD*. S stands for proteins precipitated from supernatant of cell surface extracts while WCP means whole cell proteins.

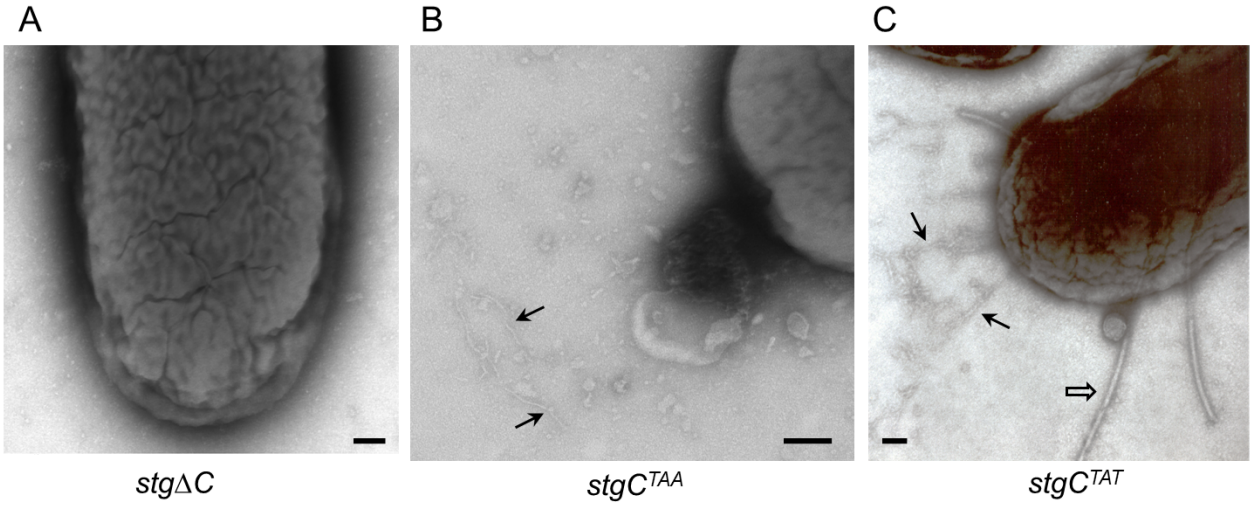


Figure 2. Observation of Stg fimbriae in ORN172 strain by transmission electron microscopy. (A) Strain harboring a deletion of *stgC* usher (*stg*ΔC) (DEF144) was used as negative control. (B) Detached fimbriae observed from strain harboring the wild-type *stg* fimbrial system ($stgC^{TAA}$) (DEF145). (C) Fimbriae at the cell surface were observed in strain harboring a modified codon ($stgC^{TAT}$) (DEF153). Black arrows show Stg fimbriae while the open arrow indicates flagella. Bars = 100 nm.

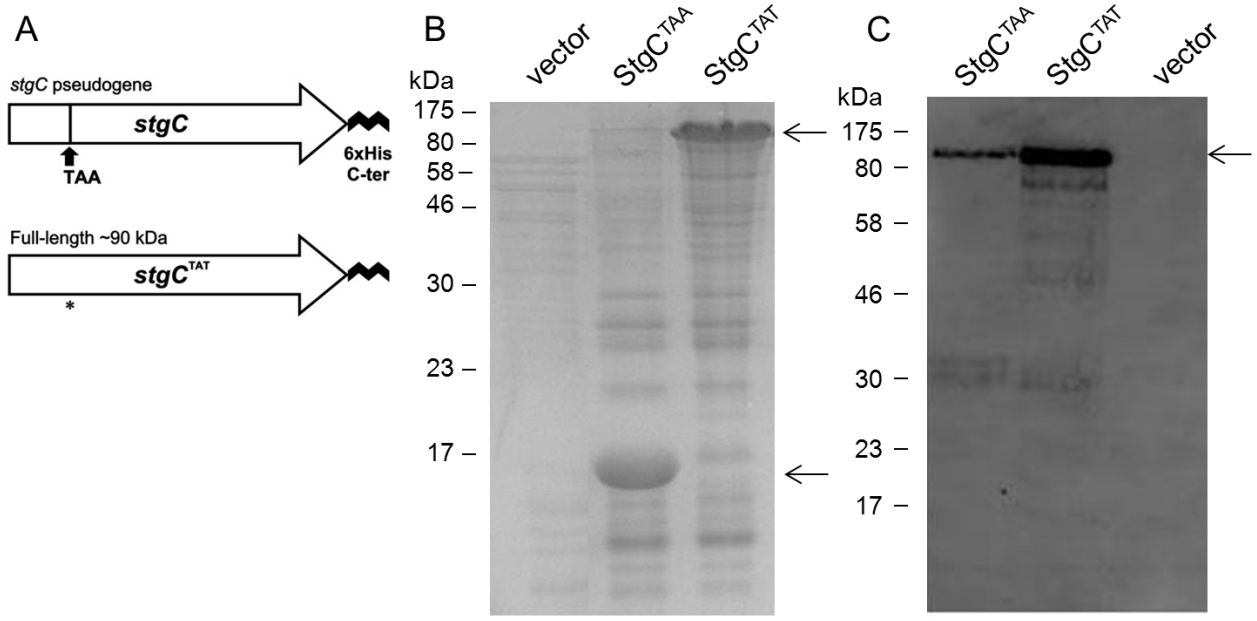


Figure 3. Protein analysis of StgC ushers harboring a 6-Histidine tag at their C-terminal end. (A) Scheme of the different versions of *stgC* usher cloned in pET21a vector. The asterisk shows the site-directed mutagenesis. (B) Coomassie-stained 15% SDS-Polyacrylamide gel after induction in *E. coli* BL21. Inclusion bodies from strains harboring the empty vector (DEF554), wild-type $StgC^{TAA}$ (DEF195) and modified $StgC^{TAT}$ (DEF196) are shown. Arrows show the 90 kDa full-length and the 16 kDa StgC ushers respectively. (C) Immunoblot with anti-6 Histidine tag antibody on inclusion bodies of vector, $StgC^{TAA}$ and $StgC^{TAT}$. The inclusion bodies from $StgC^{TAT}$ were diluted 1/10 to obtain a band not too strong for the detection.

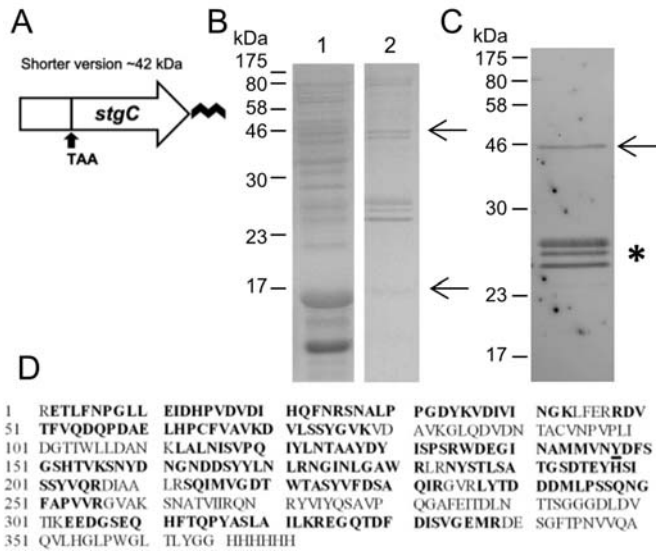


Figure 4. Protein analysis of StgC$^{42\ kDa}$. (A) Schematic representation of the 6-His tagged shorter usher. (B) Coomassie-stained 15% SDS-polyacrylamide gel of proteins obtained from inclusion bodies after induction (lane 1) or purified with Ni-NTA beads (lane 2). Arrows show the truncated 42 kDa StgC and the 16 kDa protein produced when translation ceased at the premature TAA stop codon. Samples were migrated on the same gel, but some wells were removed from the final figure. (C) Immunoblot with anti-6 Histidine tag antibody on purified proteins from StgC$^{42\ kDa}$. (D) Amino acid sequence of the predicted StgC$^{42\ kDa}$. Peptides obtained from the purified StgC$^{42\ kDa}$ identified by LC-MS/MS are shown in bold. The amino acid tyrosine (Y) inserted at the leaky stop codon is underlined.

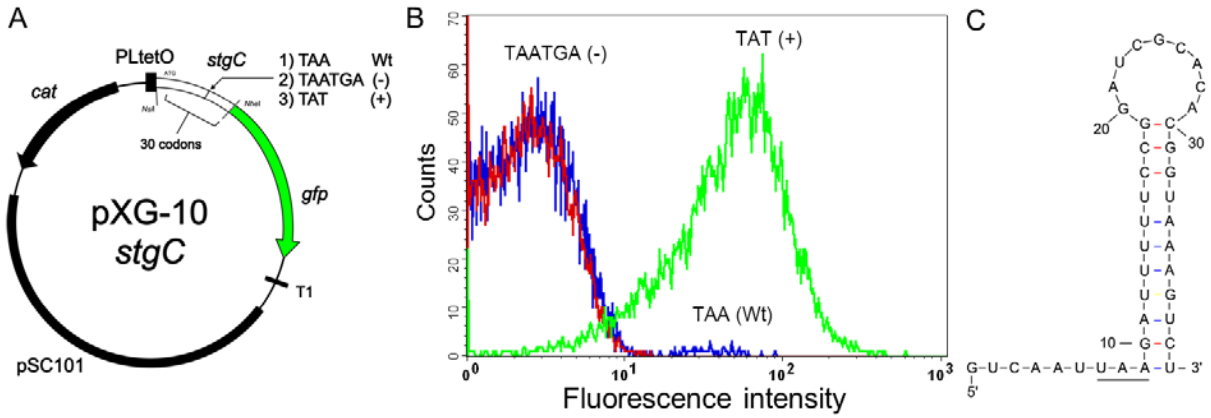


Figure 5. Quantification of *stgC* TAA readthrough in *S.* Typhi. (A) Scheme of the three different sequences of *stgC* cloned as translational fusions with GFP in pXG-10 vector. *stgC* sequence is shown in white and the position of the three modifications is indicated with an arrow. (B) Fluorescence intensities obtained from *S.* Typhi strains harboring the translational fusions between *stgC* and *gfp*. The positive control (TAT) is shown in green, the negative control (TAATGA) in red and readthrough of *stgC* pseudogene (TAA) is shown in blue. One representative experiment is shown. (C) Predicted stem-loop structure of *stgC* mRNA obtained with Mfold program. The strongest interactions are shown in red followed by blue and the weakest are in yellow. The premature stop codon is underlined.

SUPPLEMENTARY DATA

Table S1. Pseudogenes harboring premature stop codon detected in the genome of the CT18 strain of *S.* Typhi.

Gene	Name	Function	Stop codon	Position of the mutation
STY0026	*bcfC*	Fimbrial usher	2X UAA	307 and 651
STY0298	-	Conserved hypothetical protein	UAG	47
STY0428	*araJ*	AraJ protein	UGA	202
STY0525	*priC*	Primosomal replication protein	UAA	22
STY0569	*ybbW*	putative allantoin permease	UAG	143
STY0590	*fimI*	fimbrin-like protein FimI	UAG	47
STY0632	*fepE*	ferric enterobactin transport protein FepE	UAG	79
STY0762	-	putative polysaccharide export ABC transporter permease protein	UGA	188
STY0833	*slrP*	Leucine-rich repeat protein SlrP	UGA, UAG	49 and 153
STY0856	*dinG*	probable ATP-dependent helicase DinG	UGA	404
STY0858	*ybiB*	putative glycosyltransferase	UAG	67
STY0987	*ycaQ*	conserved hypothetical protein	UAG	241
STY1195	*yceJ*	cytochrome b561 homolog 2	UAG	47
STY1295	*ychJ*	conserved hypothetical protein	UAG	85
STY1525	*hyaB2*	uptake hydrogenase large subunit	UAA	178
STY1827	*yeaD*	conserved hypothetical protein	UAG	50
STY1834	*yeaJ*	conserved hypothetical membrane protein	UAA	58
STY1913	-	hydrogenase-1 small subunit	UAG	167
STY1924	*treA*	periplasmic trehalase	UAG	92
STY1995	-	transposase	UAG	42
STY2118	-	penicillin-binding protein	UAG	390
STY2196	-	conserved hypothetical integral membrane protein	UGA	85
STY2268	*dacD*	penicillin-binding protein	UGA	318
STY2398	*pbpG*	penicillin-binding protein	UGA	5
STY2432	-	putative transport protein	UGA	298
STY2501	-	putative transmembrane transport protein	UGA	85
STY2732	-	conserved hypothetical protein	UGA	128
STY2762	*sinH*	putative intimin	UAG	84

STY2901	-	putative cation transporter	UAG	24
STY2915	*ygaU*	conserved hypothetical protein	UAG	32
STY2926	-	putative transcriptional regulator	UAG	136
STY3084	*steA*	fimbrial subunit	UAG	88
STY3169	-	conserved hypothetical protein	UGA	110
STY3325	-	methyl-accepting chemotaxis protein	UAG	432
STY3338	-	possible oxidoreductase	UAG	175
STY3439	-	putative sugar kinase	UAG	334
STY3857	*yihS*	conserved hypothetical protein	UAA	379
STY3867	-	hypothetical protein	UAG	79
STY3889	*yieO*	putative transmembrane efflux protein	UAG	161
STY3920	*stgC*	fimbrial membrane usher protein	UAA	171
STY3974	*yidH*	conserved hypothetical protein	UAG	25
STY3983	-	putative membrane protein	UAG	117
STY4003	-	putative sugar-bisphosphate aldolase	UGA	68
STY4037	-	conserved hypothetical protein	UGA	190
STY4097	*gudP*	probable glucarate transporter	UAG	133
STY4244	*livJ*	amino acid-binding protein	UAG	145
STY4329	*yhfK*	conserved hypothetical protein	UGA	154
STY4506	*dmsB*	anaerobic dimethyl sulfoxide reductase chain B	UGA	132
STY4537	-	conserved hypothetical protein	UGA	203
STY4728	*yjfJ*	conserved hypothetical protein	UAG	184
STY4883	*hsdM*	subunit M of type I restriction - modification system	UAG	466

Table S2. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
Escherichia coli		
BL21 (DE3)	F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-), with DE3, a prophage carrying the T7 RNA *pol* gene	Novagen
DEF144	ORN172(pSIF063), Δ*stgC*, Kn^r, Ap^r	This study
DEF145	ORN172(pSIF065), *stg* wild-type, Kn^r, Ap^r	This study
DEF153	ORN172(pSIF067), *stgC*^{TAT}, Kn^r, Ap^r	This study
DEF188	ORN172(pSIF083), Δ*stgA*, Kn^r, Ap^r	This study
DEF189	ORN172(pSIF084), Δ*stgD*, Kn^r, Ap^r	This study
DEF195	BL21(pSIF110), (StgC), Ap^r	This study
DEF196	BL21(pSIF111), (StgC^{TAT}), Ap^r	This study
DEF197	BL21(pSIF169), (StgC$^{42\ kDa}$), Ap^r	This study
DEF218	BL21(pSIF090), (StgD), Ap^r	This study
DEF489	ORN172(pSIF063+pSIF125), Δ*stgC* + *stgC*, Kn^r, Ap^r, Cm^r	This study
DEF554	BL21(pET-21a)	This study
ORN172	*thr-l leuB thi-1 Δ(argF-lac)U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44* Δ(*fimBEACDFGH*)::*kan pilG1*	(1)
S. Typhi		
ISP1820	Wild-type *S*. Typhi	(2)
DEF595	ISP1820(pSIF201), *stgC*^{TAA}-*gfp*	This study
DEF596	ISP1820(pSIF205), *stgC*^{TAT}-*gfp*	This study
DEF667	ISP1820(pSIF228), *stgC*^{TAATGA}-*gfp*	This study
Plasmids		
pBAD24	Arabinose-inducible vector, Ap^r	(3)
pBAD33	Arabinose-inducible vector, Cm^r	(3)
pET-21a	IPTG-inducible T7 RNA-polymerase dependent expression vector for high-level expression of a recombinant protein fused to the 6xHis-Tag at C-terminus, Ap^r	Novagen
pSIF063	pBAD24 carrying fragment of *stgABD* from *S*. Typhi (Δ*stgC*)	This study
pSIF065	pBAD24 carrying wild-type fragment of *stgABCD* from *S*. Typhi	This study
pSIF067	pBAD24 carrying a site-directed mutagenesis at the 171st codon of *stgABCD* from *S*. Typhi changing the TAA stop codon for a TAT codon (*stgC*^{TAT})	This study
pSIF083	pBAD24 carrying fragment of *stgBCD* from *S*. Typhi	This study

	(Δ*stgA*)	
pSIF084	pBAD24 carrying fragment of *stgABC* from *S*. Typhi (Δ*stgD*)	This study
pSIF090	pET-21a carrying fragment of *stgD* without its signal sequence from *S*. Typhi for antibody production	This study
pSIF110	pET-21a carrying fragment of *stgC* from *S*. Typhi without its signal sequence	This study
pSIF111	pET-21a carrying fragment of $stgC^{TAT}$ from *S*. Typhi without its signal sequence	This study
pSIF125	pBAD33 carrying wild-type *stgC* from its start to its stop codon for complementation	This study
pSIF169	pET-21a carrying a shorter fragment of *stgC* from *S*. Typhi without its signal sequence and truncated at its C-terminus to obtain a 42 kDa protein	This study
pSIF201	Translational fusion between residues 151 to 181 of *stgC* and *gfp* harboring pBAD18s +1 site to its ATG in pXG-10 vector	This study
pSIF205	Translational fusion between residues 151 to 181 of $stgC^{TAT}$ and *gfp* harboring pBAD18s +1 site to its ATG in pXG-10 vector. Used as positive control.	This study
pSIF228	Translational fusion between residues 151 to 181 of $stgC^{TAATGA}$ and *gfp* harboring pBAD18s +1 site to its ATG in pXG-10 vector. Used as negative control.	This study
pXG-10	Translational fusion vector with *gfp*, Cm^r	(4)

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Table S3. Primers used in this study

Primers*	Sequence	Used to generate construct
stgAF noprom	GGAATTCCAATAGAGTTATAATGAAACTGAA	All constructs inserted in pBAD24
stgAF over	*TCAAAACGT*CGAAGCTTCCAACAGCGA	pSIF083
stgAR over	*GAAGCTTCG*ACGTTTTGATCAGCGGTTTG	pSIF083
stgCF-*Nde*I	GGAATTCCATATGAGGGAAACGCTATTTAACCCT G	pSIF110, pSIF111, pSIF169
stgC'R 21	CCGCTCGAGTTGGCAAGTGACGGCTTGCT	pSIF110, pSIF111
stgCF-*Xba*I RBS	GCTCTAGATAAGGAGGTCTTGATGATTCACCCT	pSIF125
stgC'R-*Hin*dIII	CCCAAGCTTTTATTGGCAAGTGACGGCTT	pSIF125
stgC overF	*GCCTCGTCT*CAGAGAATGCTGAACTGGTGC	pSIF063
stgC overR	*CATTCTCTG*AGACGAGGCAAAGACGAGGG	pSIF063
stgC TAT F	*ATGATGGTCAATTATGATTTTTCCGGATCG*	pSIF067
stgC TAT R	*CGATCCGGAAAATCATAATTGACCATCAT*	pSIF067
stgC40R	CCGCTCGAGGCCGCCATAGAGCGTTAATC	pSIF169
stgCF *Nsi*I	GTTTTTATGCAT**ACCCGTTTTTTTGGATGGAGTG AAACGATG**TATGACTATATCAGCCCGTCA	pSIF201, pSIF205, pSIF228
stgCR *Nhe*I	GTTTTTGCTAGCAGACTTTACCGTGTGCGATCC	pSIF201, pSIF205, pSIF228
stgCF TGA	TGATGGTCAAT*TAATGA*GATTTTTCCGGATCGC	pSIF228
stgCR TGA	TCCGGAAAAATC*TCATTA*TTGACCATCATCGC	pSIF228
stgDF-*Nde*I no SS	GGAATTCCATATGGGCGATGGCATTTGTCATGC	pSIF090
stgDR-*Xho*I	CCGCTCGAGGTTCGTTATTTCCAGCGTGA	pSIF090
stgDF over	*CTGGAGACA*CGGATGGTTAAGGTGACAG	pSIF084
stgDR over	*AACCATCCG*TGTCTCCAGATCCCTGACCT	pSIF084
stgDR	GCTCTAGACATTGATATGACTTATTTTG	pSIF063, pSIF065
stgDR2	GCTCTAGAGTAGTTCATTGATATGACTT	pSIF067
stgDR3	GCTCTAGAAACCTGGGATGGTTTGATTTC	pSIF083, pSIF084

*Restriction enzyme sites are underlined. Letters in italics denote overlapping sequences.

pBAD18s +1 sequence to the ATG is shown in bold letters.

CHAPITRE 4 – Discussion

Les connaissances sur la pathogenèse *S.* Typhi causant la fièvre typhoïde chez l'humain sont extrapolées principalement du modèle de fièvre entérique de *S.* Typhimurium chez la souris. Ce modèle a permis de découvrir des déterminants génétiques essentiels lors de la pathogenèse communs à ces deux sérovars. Néanmoins, il existe certaines différences dans les fonctions des facteurs de virulence (71, 213). Il s'avère crucial d'étudier l'implication dans la pathogenèse des gènes de virulence uniques au sérovar Typhi. Lors de cette étude, la caractérisation du fimbria Stg, retrouvé uniquement chez le sérovar Typhi de l'espèce *S. enterica*, a été effectuée. Le rôle de Stg lors de l'interaction avec des cellules humaines et le mécanisme permettant sa biogenèse ont été étudiés.

4.1. Régulation des fimbriae

Dans la première partie de ce projet, les conditions de croissance optimales pour l'expression de *stg* ont été évaluées grâce à une fusion transcriptionnelle entre le promoteur de *stgA* et le gène rapporteur *lacZ.* Cette fusion a été intégrée dans le chromosome de *S.* Typhi par recombinaison homologue pour créer la souche DEF068 (ISP1820 :: P*stgA ::lacZ)*. La croissance dans un milieu pauvre et sur une surface solide représentent les deux conditions de croissance testées les plus favorables pour l'expression de *stg*. Les niveaux d'expression obtenus peuvent être considérés comme étant faibles en comparaison d'autres gènes mesurés avec le même système intégré qui peuvent produire jusqu'à 4000 unités Miller (94). Cependant, un point commun émerge lorsque les profils d'expression des fimbriae sont comparés entre *E. coli* et *Salmonella* : le seul fimbria fortement exprimé dans les milieux de culture conventionnels est le fimbria de type 1 (voir Chapitre 1, section 1.4, Fig. 2) (105, 139). Ces bactéries possèdent chacune une douzaine de fimbriae et la majorité d'entre eux sont exprimés seulement lorsqu'elles infectent leur hôte. Par exemple, la croissance de *S.* Typhimurium à l'intérieur de boucles iléales ligaturées a favorisé l'expression significative de 8 fimbriae autres que *fim* (105). Le ou les facteurs permettant cette activation fimbriaire strictement dans l'hôte sont actuellement inconnus. La présence

de molécules sécrétées ou retrouvées dans l'hôte pourrait être responsable de cette activation.

En plus d'étudier les signaux environnementaux pouvant mener à l'expression fimbriaire, l'identification de régulateurs transcriptionnels peut fournir des indices sur le rôle des fimbriae. Lorsque les régulateurs sont situés près de l'opéron fimbriaire, leur identification en est facilitée. Aucun gène codant pour un régulateur potentiel n'est retrouvé près de la séquence nucléotidique de *stg*, ni aucune séquence GATC permettant la méthylation de l'ADN. La variation de phase a été évaluée en effectuant plusieurs séries d'étalement de la souche DEF068 sur géloses LB renfermant du X-gal, mais aucune variation dans l'expression n'a été observée. Ainsi, la recherche de régulateurs transcriptionnels de l'opéron *stg* a donc été entreprise. L'approche utilisée consistait à effectuer des mutations aléatoires avec le transposon mini-Tn*5* Km (46). La quantification de l'activité β-galactosidase a été réalisée sur les colonies présentant une forte coloration rose sur gélose MacConkey. Certains clones présentaient des valeurs de plus de 1000 unités Miller comparativement à 30 unités Miller pour la souche DEF068. Le séquençage des clones a révélé l'insertion du transposon exclusivement dans la région promotrice de *stgA*. Ainsi, le promoteur de la cassette Km du transposon devait être responsable de cette forte activation.

Pour trouver les régulateurs négatifs de *stg*, une technique récente appelée SIMPLE (*Screening with Immunomagnetic Particles for Ligand Expression*) et mise au point avec *S.* Typhimurium pourrait être utilisée (174). Le principe consiste à capturer les bactéries qui expriment les fimbriae de façon constitutive dans une banque de mutants générée à l'aide d'un transposon. La capture s'effectue avec un anticorps spécifique dirigé contre la sous-unité majeure du fimbria et couplé à des billes magnétiques. Les mutants sélectionnés sont cultivés et la sélection est répétée au moins deux fois pour amplifier la banque. L'expression du fimbria est confirmée par Western blot et les mutants positifs sont séquencés pour identifier le régulateur négatif inactivé par le transposon. Pour effectuer cette technique avec *stg*, il faudrait produire un anticorps dirigé contre StgA au lieu de

StgD, puisque la sous-unité majeure est retrouvée en plusieurs copies dans le filament, contrairement à l'adhésine, et facilite la récolte des mutants.

L'utilisation du programme de prédiction bioinformatique Virtual Footprint a prédit des sites de liaison pour les protéines régulatrices H-NS et IHF exactement entre la région -35 et -10 putative de *stgA* (158). Cette prédiction constitue une donnée extrêmement intéressante. En effet, il est reconnu que l'expression de *hilA*, le régulateur global du SST3-1 utilisé pour l'invasion intestinale, est réprimée par H-NS et qu'IHF atténue cette répression lors de l'entrée en phase stationnaire (203). Par conséquent, l'expression de *stg* pourrait être jumelée à celle du SST3-1 et permettre une interaction efficace avec la barrière intestinale. Cette information suggère que H-NS et IHF pourraient être les régulateurs responsables de l'activation fimbriaire principalement à l'intérieur de l'hôte. H-NS est déjà reconnu comme étant le régulateur négatif de la majorité des facteurs de virulence de *Salmonella* (140). Une étude d'immunoprécipitation de la chromatine a révélé la liaison de H-NS sur l'ADN de 11 des 12 fimbriae situés sur le chromosome de *S.* Typhimurium (140). Cette expérience devrait être effectuée avec IHF pour confirmer qu'il constitue un régulateur positif des facteurs de virulence de *Salmonella*, voire même de toutes les entérobactéries. Une molécule provoquant l'inhibition de la transcription d'IHF et d'autres régulateurs globaux a déjà été testée et s'avère efficace contre *E. coli* (44). Il s'agit d'un composé de ruthénium carbonylé [$Ru(CO_3)Cl$(glycinate)] qui pénètre à l'intérieur des bactéries et libère rapidement du monoxyde de carbone (CO) (44). Parmi ses effets pouvant expliquer les modifications transcriptionnelles, nottons la production de dérivés réactifs de l'oxygène, l'inhibition de la respiration bactérienne et la destruction des protéines fer-souffre (244). Cette molécule a même permis la survie de souris infectées à *Pseudomonas aeruginosa*, suggérant une possibilité d'utilisation contre les infections à *Salmonella* (49).

4.2. Implication de *stg* dans la virulence de *S.* Typhi

Lors de l'interaction avec des cellules humaines, les deux phénotypes observés en présence de *stg* sont une augmentation de l'adhérence aux cellules épithéliales, ainsi qu'une

diminution de la phagocytose par les macrophages (Chapitre 2). En considérant ces informations ainsi que les données obtenues avec la fusion transcriptionnelle, il est possible d'établir un lien dans la pathogenèse de *S*. Typhi. Puisque *stg* ne semble pas être induit par un pH acide, il s'avère peu probable qu'il soit requis lors du passage dans l'estomac (données non montrées). Aucune induction n'a été observée en présence de bile, suggérant aussi une absence d'utilisation au niveau de l'interaction avec les cellules du foie ou de la vésicule biliaire. Il existe une bonne homologie entre Stg et le fimbria Lpf2 retrouvé chez *E. coli* O157:H7 (Chapitre 2). Il a été démontré que Lpf2 est utilisé lors de l'interaction précoce avec les cellules intestinales humaines (252). De plus, selon l'homologie observée entre les placiers, *stgC* et *lpf2C* font partie du groupe γ-1, où plusieurs fimbriae requis pour la colonisation intestinale sont retrouvés (13, 173, 251, 262). Il y a donc une forte possibilité que Stg soit aussi utilisé lors de l'interaction initiale avec les cellules intestinales humaines. Ces informations n'empêchent pas qu'il puisse jouer un rôle plus tard dans l'infection, par exemple lors de la phase chronique.

Les fimbriae possèdent généralement une spécificité de liaison envers une molécule de l'hôte et effectuent des interactions avec d'autres protéines, des hydrates de carbone ou avec des lipides (176). Plusieurs adhésines fimbriaires interagissent avec les constituants de la matrice extracellulaire, tels la fibronectine, la laminine et le collagène, des protéines glycosylées formant une couche sur les cellules intestinales (176). L'identification du récepteur reconnu par les fimbriae Stg permettrait de mieux comprendre le moment précis de son utilisation dans la pathogenèse. Puisque notre laboratoire possède maintenant la protéine StgD purifiée, ainsi qu'un anticorps la reconnaissant, il serait possible d'évaluer son interaction avec différents composés par le biais d'un ELISA (*Enzyme-linked immunosorbent assay*). Les fimbriae sont souvent reconnus pour lier différents saccharides. Récemment, l'utilisation de la glycomique a permis d'identifier le récepteur du fimbria Pef de *S*. Typhimurium (32). Cette technique ressemble à l'utilisation de biopuces, mais au lieu de retrouver de l'ADN sur celle-ci, 99 hydrates de carbone différents sont fixés dans le fond d'une plaque opaque. Il suffit de mettre la protéine à l'étude en contact avec ces sucres. La spécificité d'interaction est déterminée par l'utilisation d'un anticorps primaire, suivi d'une

détection à l'aide d'un anticorps secondaire qui est couplé à une molécule fluorescente. Ces expériences devraient aussi être réalisées avec la sous-unité majeure StgA du fimbria, puisqu'elle peut aussi contribuer à la spécificité d'attachement (45, 273).

Les fimbriae sont généralement utilisés lors de l'interaction avec les cellules épithéliales, mais leur implication directe avec les cellules du système immunitaire ne fait plus aucun doute (126). Ainsi, en plus d'être requis pour l'adhésion aux cellules épithéliales, Stg possède une activité anti-phagocytaire envers les macrophages. L'utilisation de cytochalasine D, un inhibiteur du cytosquelette d'actine, permet de conclure que cette diminution de phagocytose n'est pas causée par un défaut d'association entre les bactéries et les macrophages (Chapitre 2, Fig. 5). Le mécanisme moléculaire exact demeure inconnu pour le moment, mais plusieurs hypothèses pourraient expliquer cette baisse de phagocytose. La présence de Stg pourrait masquer une autre structure de surface normalement reconnue par les macrophages pour stimuler l'activité phagocytaire. Stg pourrait aussi interagir directement avec une molécule impliquée dans l'activation du système immunitaire. Par exemple, les fimbriae Psa de *Yersinia pestis* lient les résidus galactosyl associés à la portion Fc des immunoglobulines G et inhibent la phagocytose (47, 104, 190, 272). Plusieurs adhésines de surface de *Streptococcus pyogenes* qui lient la fibronectine sont associées à une activité anti-phagocytique (53, 107, 268). Une de ces études suggère la théorie du mimétisme moléculaire pour expliquer ce phénomène : en liant la fibronectine, le collagène s'accumule sur la bactérie et empêche sa reconnaissance par les cellules polymorphonucléaires de l'hôte (53). Ainsi, Stg pourrait lier des protéines de l'hôte, par exemple un composé sérique, avant d'interagir avec les macrophages et diminuer la reconnaissance de *S.* Typhi. Plusieurs protéines de surface de *Salmonella* impliqués dans l'adhérence intestinale, comme Csg, MisL et ShdA, lient la fibronectine (38, 56, 121). Il serait intéressant de vérifier si la capacité de liaison de la fibronectine constitue la cause de l'inhibition de la phagocytose. L'activité anti-phagocytaire de Stg pourrait être utilisée par *S.* Typhi pour effectuer une propagation systémique efficace ou permettre le développement initial d'un biofilm en empêchant son élimination par les macrophages.

4.3. Fonctionnalité du placier StgC

La deuxième partie de ce projet consistait à déterminer le mécanisme permettant la fonctionnalité des fimbriae Stg, malgré la présence d'un codon d'arrêt prématuré dans le gène *stgC* codant pour le placier. L'hypothèse qui semblait la plus plausible concernant la fonctionnalité de placiers possédant des codons d'arrêt, tels que *stgC* et *lpf1C*, était qu'un phénomène de complémentation par un autre placier devait avoir lieu (139), Chapitre 2). Les placiers recrutent généralement leurs sous-unités fimbriaires de façon très spécifique (212, 222), mais certaines études ont confirmé la possibilité de complémentation entre deux systèmes différents (246). La souche afimbriaire ORN172, dérivée d'*E. coli* K-12, possède une inactivation seulement au niveau de son fimbria de type 1 et possède 11 autres systèmes fimbriaires putatifs dans son chromosome qui pourraient complémenter *stgC* (127, 266). Afin de vérifier cette hypothèse, il fallait tout d'abord forcer la transcription de l'opéron *stgABCD* dans un milieu conventionnel. Le clonage de *stg* sous le contrôle de son promoteur dans le plasmide pWSK29, retrouvé de 3 à 5 copies par bactérie, n'est pas suffisant pour détecter StgD de façon efficace. Ainsi, toutes les modifications introduites dans *stg* ont été mises sous le contrôle d'un promoteur inductible à l'arabinose. La souche possédant l'opéron sauvage renfermant le pseudogène a permis d'observer l'exportation efficace de StgD à la surface de la bactérie par Western blot. Puisque *stgD* est sous le contrôle de son propre site de liaison au ribosome, cette expérience permet de conclure que l'augmentation de la transcription de l'opéron est suffisante pour obtenir une production fimbriaire détectable chez *E. coli*.

La délétion non-polaire complète de *stgC* résulte en l'accumulation de StgD à l'intérieur de la souche ORN172 d'*E. coli* (Chapitre 3, Fig. 1B). Cette expérience constitue la preuve que les fimbriae Stg requièrent spécifiquement leur placier pour être exportés à la surface. La souche d'*E. coli* arborant la modification du codon d'arrêt prématuré de *stgC* pour un codon insérant une tyrosine démontrait parfois une augmentation dans l'exportation de StgD. En comparant 4 extractions différentes de protéines de surface, deux membranes ont clairement démontré une plus forte concentration de StgD dans les extraits

d'*E. coli* comportant le placier $StgC^{TAT}$ par rapport à la souche renfermant $StgC^{TAA}$. Néanmoins, l'analyse de tous ces résultats n'a pas démontré de différence significative globale. Cette observation peut être expliquée par le fait que s'il y a davantage de placiers complets traduits pour la souche renfermant $StgC^{TAT}$, il peut y avoir davantage de fimbriae par bactérie. De plus, si les ribosomes traduisent entièrement *stgC*, ils protègent l'ARNm contre les RNAses et stabilisent les transcrits.

Les sous-unités StgD nécessitent la présence du placier StgC pour leur exportation. Il est possible que les 170 acides aminés traduits avant le codon d'arrêt prématuré soient suffisants pour médier l'exportation de StgD. Un alignement réalisé entre les 170 aa situés en N-terminal de StgC et la séquence du placier FimD récemment cristallisé (194), permet de remarquer que seule la traduction du domaine N-terminal (NTD) s'effectue s'il y a un arrêt de traduction au codon stop prématuré (voir Chapitre 1, Fig. 1.6). En cas d'arrêt prématuré de traduction, il n'y a donc pas de pore produit pour StgC. Sachant que les placiers fonctionnent en dimères, la partie NTD de StgC pourrait présenter ses sous-unités fimbriaires au placier d'un autre système et utiliser ce pore pour exporter les sous-unités StgA et StgD. Pour tester cette hypothèse, la délétion non-polaire des résidus 2 à 202 de la protéine mature StgC a été effectuée, puis tranformée dans la souche ORN172 d'*E. coli*. La présence de StgD a été vérifiée dans les extraits de protéines de surface. Les sous-unités StgD ont été exportées à la surface d'*E. coli* ORN172, malgré la délétion de la partie NTD de StgC (voir Annexe II, Figure S1). Ce résultat prouve l'importance de la partie située en aval du codon d'arrêt prématuré dans la fonctionnalité du placier StgC chez *E. coli*. Un placier PapC renfermant une délétion des résidus 2 à 11 n'interagit pas correctement avec ses sous-unités et empêche sa fonction dans la biogenèse fimbriaire (165). Puisque chaque système fimbriaire possède son propre placier et qu'il en existe différentes classes, ce résultat suggère qu'il pourrait y avoir des différences entre les interactions du complexe chaperonne/sous-unité au placier selon les systèmes. La majorité des études ont été réalisées avec les placiers PapC et FimD d'*E. coli*, il serait pertinent d'évaluer la fonction et l'importance de la partie N-terminale de placiers chez d'autres fimbriae.

Les résultats obtenus par Western blot ont été confirmés par de la microscopie électronique à transmission. Des minuscules fibrilles ont été observées chez la souche d'*E. coli* possédant l'opéron *stgABCD* sauvage, ainsi que celle possédant le codon d'arrêt modifié. La présence de longs filaments sur les bactéries suggère que la sous-unité StgA est aussi exportée efficacement par StgC. Avec le fimbria de type 1, il s'agit du 2^{e} exemple de fimbriae exprimés par la voie chaperonne/placier de *S.* Typhi à être visualisé (159). Certaines difficultés ont cependant été rencontrées lors de cette expérience. Tout d'abord, le marquage des fimbriae à l'aide de l'anticorps dirigé contre StgD et un anticorps secondaire couplé à des billes d'or a été tenté. Puisque StgD est retrouvé en une seule copie par filament, cette approche n'a pas permis de marquer les fimbriae de façon évidente. De plus, les fimbriae se détachaient très facilement des bactéries, même lors des pipettages. Très peu de fimbriae attachés aux bactéries ont été observés, même pour la souche qui contient le placier *stgC* sans codon d'arrêt prématuré. Un temps d'induction avec de l'arabinose trop long, combiné à une centrifugation pour mettre les bactéries sur les grilles, expliquent possiblement la difficulté d'observer les structures sur les bactéries. Il faudrait essayer de faire coller les bactéries sur les grilles en mettant celles-ci directement dans une goutte de culture induite. Cependant, cette technique augmente les impuretés et peut nuire à la prise de photos.

L'insertion d'une étiquette 6-histidines en C-terminal de StgC a fourni de précieuses informations quant au mécanisme impliqué dans sa fonctionnalité. Cette expérience a non seulement permis d'observer la traduction complète de *stgC* malgré le codon d'arrêt prématuré, mais elle a aussi démontré la suppression du codon d'arrêt.

4.4. Suppression des codons d'arrêts

Le séquençage de StgC produit dans *E. coli* BL21 a révélé l'insertion d'une tyrosine au codon d'arrêt prématuré TAA et a confirmé l'insertion des autres acides aminés retrouvés en aval de celui-ci. Ce résultat est d'autant plus intéressant que seule la glutamine avait été retrouvée à un codon TAA dans une souche d'*E. coli* sans ARNt suppresseur

(167). Le phénomène cellulaire permettant l'insertion d'une tyrosine au codon d'arrêt TAA n'est pas encore déterminé. Il pourrait s'agir d'une erreur lors de la réplication, de la transcription ou de la traduction. Chez les bactéries, le taux d'erreur par nucléotide est normalement de 10^{-8} à 10^{-9} pour la réplication, de 10^{-4} à 10^{-5} lors de la transcription, alors qu'il s'élève à 10^{-3} à 10^{-4} par acide aminé lors de la traduction (151). Les résultats obtenus avec la fusion traductionnelle de *stgC* insérée dans *S.* Typhi suggèrent qu'un autre facteur permet d'augmenter ce taux d'erreur jusqu'à 10^{-2}. La suppression d'un codon d'arrêt TAA est normalement un processus rare, puisque les deux facteurs de relâche peuvent le reconnaître et arrêter la traduction (226).

Puisque *S.* Typhi possède une forte proportion de gènes inactivés par un codon d'arrêt prématuré, l'hypothèse que cette bactérie effectue un taux d'erreurs de traduction plus élevé a été testée. Pour y arriver, le système développé par l'équipe du Dr. Michael Yarus a été utilisé (223). Il s'agit de trois plasmides renfermant chacun le gène de la luciférase de *Vibrio harveyi* inactivé au 13^{e} codon par un des trois codons d'arrêt. Ceux-ci ont été électroporés dans la souche sauvage ISP1820 de *S.* Typhi et l'activité de la luciférase a été comparée de façon qualitative avec *E. coli* K12 (voir Annexe II, Fig. S2). Lorsque la suppression du codon d'arrêt survient, il y a production de luciférase et émission de lumière. Un film a été placé sur la gélose striée par les différents mutants à tester pour comparer la production de luminosité par la luciférase. Plusieurs informations intéressantes ont été obtenues de cette expérience. Retrouvés dans un même contexte nucléotidique, la suppression des codons d'arrêts survient de la même façon entre *E. coli* et *S.* Typhi, soit TGA > TAG > TAA. De façon générale, *S.* Typhi ne semble pas effectuer une plus grande suppression des codons d'arrêt qu'*E. coli*, malgré une forte prévalence de pseudogènes dans son génome.

Plusieurs phénomènes permettent d'effectuer la suppression des codons d'arrêt lors de la traduction chez les bactéries, comme par exemple les ARNt suppresseurs ou natifs, les protéines et les ARN ribosomaux et le contexte nucléotidique du codon d'arrêt (64). Le seul exemple connu de suppression d'un codon d'arrêt dans un gène de virulence chez les

eubactéries s'effectue par un ARNt suppresseur (109). La vérification de l'acide aminé inséré au codon TAA par *S.* Typhi permettrait de déterminer si un ARNt suppresseur précis est impliqué pour *stgC*. En effet, deux copies chromosomiques sont requises pour effectuer la suppression de cette façon, une copie sauvage et une autre mutée dans son anticodon. *S.* Typhi ne comporte qu'un seul ARNt natif insérant la tyrosine. Ainsi, il ne peut pas effectuer la suppression du TAA par un ARNt suppresseur si une tyrosine est aussi retrouvée. Certaines modifications au niveau des bases de l'ARN peuvent provoquer un changement dans l'appariement et influencer l'insertion des acides aminés. Les ARNt sont reconnus pour être modifiés de façon post-transcriptionnelle à plusieurs positions et plusieurs nucléosides modifiés ont été localisés à la 1ère base de l'anticodon (2). En 1966, Crick a formulé l'hypothèse que lors de la traduction, les deux premières bases du codon effectuent des interactions canoniques purine-pyrimidine, alors que la 3^{e} base peut parfois s'apparier sans suivre cette loi (41). C'est à cette position « Wobble » qu'il y aurait un mésappariement A-G au niveau du codon d'arrêt de *stgC* (voir Fig. 4.1). Une modification complexe de la guanosine produit de la queuosine qui peut s'apparier avec la cytosine et l'uracile (168). Cette modification ne peut donc pas intervenir pour *stgC*, puisque une adénosine doit s'apparier à cet endroit. Théoriquement, une simple réaction de désamination de la guanosine peut mener à la formation d'inosine pouvant s'apparier avec l'adénine, la cytosine ou l'uracile (15). Cependant, aucune enzyme cellulaire effectuant cette réaction n'a été identifiée (135).

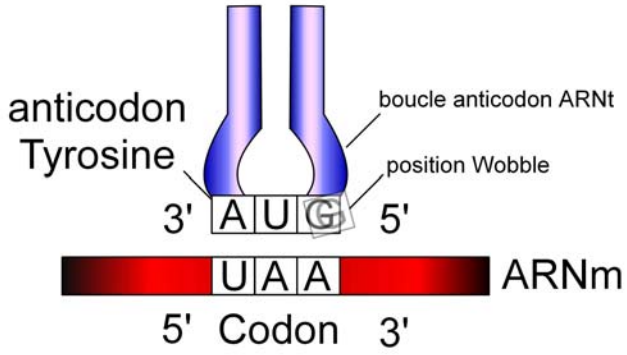

Figure 4.1. Appariement de type « Wooble ». Chez *S.* Typhi, le seul ARNt^{tyr} retrouvé possède la séquence GUA au niveau de son anticodon. Un mésappariement à la position Wobble pourrait permettre l'insertion d'une tyrosine.

Les protéines ribosomales généralement impliquées dans les erreurs de traduction sont RpsD et RpsL. Une mutation dans RpsD engendre une augmentation dans le taux d'erreurs de traduction, alors qu'une mutation dans RpsL augmente la fidélité de la traduction lors du processus de sélection des ARNt (129). La souche ORN172 possède la mutation *rpsL135* qui modifie le 45^{e} acide aminé et provoque la résistance à la streptomycine (22, 181, 226, 266). Il a été démontré que la modification du 45^{e} acide aminé de RpsL augmente la fidélité de traduction comparé à la souche sauvage, mais à des niveaux intermédiaires par rapport à d'autres mutations effectuées dans *rpsL* (229). Puisque la fonctionnalité du placier StgC a été prouvée dans cette souche, cela suggère que le phénomène de suppression est indépendant des protéines ribosomales.

Ainsi, le contexte nucléotidique semble l'explication la plus probable de la suppression dans *stgC*. Chez *E. coli*, l'efficacité de terminaison de la traduction n'est que de 50% lorsqu'il y a présence d'une guanine en 3' du codon d'arrêt TAA, comme pour *stgC* (243). De plus, une structure en épingle à cheveux a été prédite par le programme Mfold directement en aval du codon d'arrêt prématuré de *stgC*. Cette structure tige-boucle est nécessaire pour promouvoir l'insertion de la sélénocystéine au niveau d'un codon d'arrêt TGA prématuré chez *E. coli* (278). Exactement comme ce qui est prédit pour *stgC*, le dernier acide nucléique du codon d'arrêt est situé directement au début de la tige-boucle.

Un alignement nucléotidique réalisé entre la séquence de *stgC* de *S.* Typhi et l'orthologue situé dans le même locus chez *S. bongori* démontre la présence du codon TAT chez ce dernier (Annexe II, Fig. S3A). Une tyrosine est donc insérée dans la protéine StgC de *S. bongori* à ce codon. L'acide nucléique retrouvé directement en 5' diffère entre ces 2 espèces, alors que 11 nucléotides situés en 3' sont identiques. De plus, la structure tige-boucle semble aussi se former dans l'ARNm de *stgC* de *S. bongori* (Annexe II, Fig. S3B). Ces observations suggèrent que la séquence située en 3' du codon d'arrêt TAA ou la structure tige-boucle dans l'ARNm de *stgC* pourrait promouvoir l'insertion de la tyrosine à ce codon chez *S.* Typhi. Pour confirmer l'hypothèse que le contexte nucléotidique permet la suppression dans *stgC*, il faudrait essayer de déstabiliser cette tige-boucle putative et comparer les niveaux de traduction à l'aide la fusion avec *gfp*. Pour ce faire, différentes mutations devraient être introduites de manière dirigée, principalement au niveau des interactions G/C de forte affinité. Les modifications générées devraient être des mutations ponctuelles qui n'influencent pas le type d'acide aminé inséré. Le codon d'arrêt pourrait aussi être déplacé après cette tige-boucle pour vérifier si le contexte général dicte entièrement la suppression du codon d'arrêt.

4.5. Rôle des pseudogènes dans la virulence

Plusieurs exemples dans la littérature permettent de croire que les pseudogènes interviennent réellement dans les fonctions physiologiques des organismes. Chez *S.* Typhi, d'autres pseudogènes semblent être impliqués directement dans sa pathogenèse. Par exemple, des anticorps dirigés contre le produit du pseudogène *shdA* et contre la sous-unité fimbriaire BcfD, qui est exportée par le placier BcfC inactivé par deux codons d'arrêt TAA, ont été retrouvés chez des patients convalescents de la fièvre typhoïde (90, 103). De plus, l'inactivation des pseudogènes *siiE*, *marT*, *yiaH*, STY4054 par l'introduction d'un transposon a diminué significativement la survie de *S.* Typhi dans le modèle de souris humanisées (134). Des peptides ont été séquencés pour les produits des pseudogènes *yifB*, *yhjL* et *livJ* prouvant leur traduction (192). Deux études ont démontré des effets

phénotypiques associés à des pseudogènes de *S*. Typhi jugés non-fonctionnels par le passé (210, 261). Des expériences plus poussées sont néanmoins nécessaires pour évaluer leurs rôles dans la virulence de *S*. Typhi et confirmer que les phénotypes observés sont reliés à la traduction complète des pseudogènes. Il serait intéressant de vérifier la traduction de ces pseudogènes à partir de la même construction utilisée pour *stgC*.

Les conditions de croissance stressantes, comme une carence en nutriment ou la présence d'antibiotiques, sont reconnues comme étant un facteur prédisposant à des erreurs de traduction (151). Ces circonstances peuvent être rencontrées lors de l'infection de l'hôte. Seules les bactéries qui effectuent la suppression du codon d'arrêt dans *stgC* peuvent exprimer le fimbria à leur surface. Ainsi, ce phénomène pourrait générer de la diversité antigénique (8) à l'intérieur d'une même population bactérienne, semblable à ce qui est observé lors de la variation de phase fimbriaire. La suppression des codons d'arrêts pourrait même être utilisée comme une forme de régulation génique chez les bactéries. En fait, il est déjà reconnu que les virus à ARN utilisent fréquemment la suppression des codons d'arrêt pour générer différentes longueurs de protéines impliquées dans leur cycle cellulaire (15). Cela permet non seulement d'optimiser l'utilisation de leur génome restreint, mais le ratio protéique, ainsi que le produit formé par cette suppression, sont généralement essentiels pour la propagation virale efficace (15).

Étonnamment, la recherche concernant la fonctionnalité des pseudogènes est encore plus avancée chez les eucaryotes, malgré la complexité de travailler avec un organisme diploïde. Plus de 20000 pseudogènes ont été recensés dans le génome humain (277). Des profils d'expression spatio-temporels différents ont été observés entre le gène sauvage et sa copie pseudogène (63, 179). De plus, des conditions physiologiques spécifiques comme le diabète (33) et le cancer (197, 240, 279) peuvent être causées par un changement significatif dans l'expression des pseudogènes. Plusieurs études ont prouvé que les transcrits de pseudogènes peuvent agir autant en tant que répresseurs et stabilisateurs de l'ARNm de leur homologue sauvage (résumé dans (196)). Tout comme pour les bactéries, la traduction des pseudogènes eucaryotes a aussi été démontrée (23, 114).

Certains antibiotiques, comme la famille des aminoglycosides, augmentent le taux d'erreurs dans la traduction et effectuent la suppression des codons d'arrêts prématurés (182). Basé sur cette observation, certaines maladies humaines associées à des pseudogènes, comme la dystrophie musculaire et le cancer, ont été traitées efficacement par l'utilisation d'antibiotiques (59, 69, 117, 118, 143). Cette information constitue la démonstration la plus éloquente de l'utilité d'étudier les pseudogènes.

CHAPITRE 5 – Conclusion

La recherche sur *S*. Typhi, l'agent étiologique de la fièvre typhoïde chez l'humain, est entravée principalement par le manque de modèles expérimentaux représentatifs et abordables. L'augmentation des isolats multi-résistants, ainsi que du nombre de cas annuel observé, constituent une menace grandissante. Il s'avère important de continuer la recherche fondamentale sur ses facteurs de virulence, que ce soit pour optimiser les vaccins existants ou trouver de nouvelles cibles thérapeutiques. Ainsi, le but de cette étude consistait à caractériser un nouveau facteur de virulence potentiel de *S*. Typhi, soit le fimbria Stg. Une caractéristique intéressante de l'opéron fimbriaire *stg* est la présence d'un codon d'arrêt prématuré TAA dans le gène *stgC* codant pour son placier, suggérant qu'il est non-fonctionnel.

Dans la première partie, les conditions d'expression de l'opéron *stg*, ainsi que son rôle dans la virulence ont été investigués. Comme la majorité des fimbriae chez les entérobactéries, l'opéron *stg* est peu exprimé dans les milieux de culture conventionnels. Des expériences effectuées à l'aide de cellules immortalisées ont permis d'observer que Stg est utilisé lors de l'adhésion sur les cellules intestinales. De plus, il agit comme une structure anti-phagocytaire lors de l'interaction avec les macrophages. Ainsi, ces données constituent la preuve que l'opéron *stg* est fonctionnel et utilisé dans la virulence. Dans la seconde partie, la fonctionnalité du placier StgC a été étudiée. Il a été démontré que la sous-unité StgD requiert la présence de son placier StgC pour être exportée à la surface des bactéries. La traduction complète du placier StgC a été confirmée et peut être réalisée grâce à l'incorporation d'une tyrosine au niveau du codon d'arrêt TAA prématuré. Pour terminer, une fusion traductionnelle a révélé que 0.8% de l'ARNm effectue la suppression du codon d'arrêt et permet la traduction complète du placier StgC chez *S*. Typhi.

Plusieurs expérimentations en lien avec ce projet pourraient être réalisées pour compléter cette étude. Tout d'abord, il serait pertinent de vérifier l'acide aminé inséré au codon d'arrêt TAA prématuré de *stgC* par une souche sauvage de *S*. Typhi. Cette information permettrait de confirmer que, même s'il n'y a qu'un seul $ARNt^{tyr}$ chez *S*.

Typhi, la suppression s'effectue par le même mécanisme que dans la souche employée d'*E. coli*. De plus, plusieurs pseudogènes de *S.* Typhi semblent être utilisés lors de sa pathogenèse (voir Discussion). Ainsi, leur rôle direct pourrait être évalué par des mutations non-polaires, puis évalué lors de l'interaction avec les types cellulaires normalement rencontrés dans le corps humain. Tout comme StgC, les protéines résultantes pourraient être étudiées avec des étiquettes positionnées en C-terminal et séquencées. Par la suite, il serait intéressant de vérifier si des déterminants génétiques communs sont responsables de l'expression de ces pseudogènes.

Il n'y a plus aucun doute que certains pseudogènes peuvent contourner les règles établies pour la traduction du code génétique et accomplir des fonctions cruciales au sein d'un organisme, même bactérien. Le prochain défi sera d'essayer de comprendre les mécanismes cellulaires impliqués dans leur fonctionnalité.

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ANNEXE I : Articles publiés non discutés dans cette thèse

Lors de cette thèse, j'ai eu l'occasion de participer à la rédaction d'une revue de littérature, ainsi que de deux articles présentant des résultats originaux, mais qui ne sont pas discutés dans cette thèse. Tous ces articles ont été soumis à un processus de révision par les pairs avant leur publication.

Liste des articles :

1. **Forest, C. G.**, Ferraro, E., Sabbagh, S. C. and F. Daigle. 2010. Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is independent of *Salmonella* pathogenicity island (SPI)-2 independent. Microbiology, **156:**3689-3698. (Co-première auteure)

2. Sabbagh, S. C., **Forest, C. G.**, Lepage, C., Leclerc, J. M. and F. Daigle. 2010. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. FEMS Microbiol. Lett., **305:**1-13.

3. Faucher, S. P., **Forest**, **C.**, Béland, M. and F. Daigle. 2009. A novel PhoP-regulated locus encoding the cytolysin ClyA and the secreted invasin TaiA of *Salmonella enterica* serovar Typhi is involved in virulence. Microbiology, **155:**477-488.

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Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is independent of *Salmonella* pathogenicity island (SPI)-2

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For successful infection, *Salmonella enterica* secretes and injects effector proteins into host cells by two distinct type three secretion systems (T3SSs) located on *Salmonella* pathogenicity islands (SPIs)-1 and -2. The SPI-2 T3SS is involved in intracellular survival of *S. enterica* serovar Typhimurium and systemic disease. As little is known regarding the function of the SPI-2 T3SS from *S. enterica* serovar Typhi, the aetiological agent of typhoid fever, we investigated its role for survival in human macrophages. Mutations in the translocon (*sseB*), basal secretion apparatus (*ssaR*) and regulator (*ssrB*) did not result in any reduction in survival under many of the conditions tested. Similar results were obtained with another *S.* Typhi strain or by using human primary cells. Results were corroborated based on complete deletion of the SPI-2 T3SS. Surprisingly, the data suggest that the SPI-2 T3SS of *S.* Typhi is not required for survival in human macrophages.

INTRODUCTION

Salmonella enterica is an important pathogen of animals and humans, which typically become infected by ingestion of contaminated food or water. There are two major groups of *S. enterica* that cause disease in humans: the systemic (typhoid fever) group and the non-typhoidal salmonellae group. Typhoid fever is mainly caused by *S. enterica* serovar Typhi (*S.* Typhi), a host-adapted serovar that is specific for humans. Non-typhoidal salmonellae are generally associated with gastroenteritis, which rarely develops into an invasive infection. *S. enterica* serovar Typhimurium is frequently associated with gastroenteritis in humans, whereas this serovar causes a systemic typhoid-like disease in susceptible mice. *S.* Typhimurium infection in susceptible mice is usually used as a model for *S.* Typhi infection in humans, as these serovars share many virulence factors, including *Salmonella* pathogenicity islands (SPIs) (McClelland *et al.*, 2001; Parkhill *et al.*, 2001; Sabbagh *et al.*, 2010).

S. Typhimurium encodes two distinct type three secretion systems (T3SSs), located on SPI-1 and SPI-2. The SPI-1 T3SS is involved in invasion of epithelial cells (Galán & Curtiss, 1989), whereas the SPI-2 T3SS is essential for survival and replication in host cells (Ochman *et al.*, 1996; Shea *et al.*, 1996). The SPI-2 T3SS corresponds to a 25 kb fragment organized in four operons: regulatory, structural I, structural II and effector/chaperone (see Supplementary Fig. S1, available with the online version of this paper, and Kuhle & Hensel, 2004). The two-component regulatory system SsrA/SsrB is responsible for the transcriptional regulation of SPI-2 operons as well as the regulation of effector genes located outside SPI-2 (reviewed by Haraga *et al.*, 2008). The T3SS needle complex comprises basal component proteins in the inner membrane (including SsaR), proto-channel proteins, outer membrane proteins, proteins forming a hollow tube generating the needle and outer ring proteins (including SseB), which form the translocon that traverses the host vacuolar membrane (Supplementary Fig. S1). This SPI-2 T3SS injects at least 20 effector proteins across the vacuolar membrane into the host cytosol (Haraga *et al.*, 2008). These effectors interact with the host machinery and are required for modification of the vacuole and modulation of intracellular processes (Brumell *et al.*, 2001; Waterman & Holden, 2003; Yu *et al.*, 2004), which will allow the bacteria to survive and replicate in a membrane-bound structure called the *Salmonella*-containing vacuole. Intracellular induction of SPI-2 genes was initially observed by using reporter assays (Valdivia & Falkow, 1997; Cirillo *et al.*, 1998; Pfeifer *et al.*, 1999) and was confirmed by microscopy (Kuhle & Hensel, 2002), microarray data (Eriksson *et al.*, 2003) and Western blotting (Coombes *et al.*, 2007).

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Abbreviations: CI, competitive index; IFN-γ, gamma interferon; MDMs, monocyte-derived macrophages; PBMCs, peripheral blood mononuclear cells; SPI, *Salmonella* pathogenicity island; T3SS, type three secretion system.

Two supplementary figures, showing the SPI-2 T3SS and the mutants generated during this study, are available with the online version of this paper.

Survival and replication of *S.* Typhimurium within cultured cells correlate with systemic survival and persistence in animal models, as mutants that fail to replicate in cultured cells are avirulent in animals (Fields *et al.*, 1986; Leung & Finlay, 1991; Shea *et al.*, 1999). Survival within macrophages is therefore an essential step for *Salmonella* pathogenesis. Two major systems are implicated in this process: (1) the two-component regulatory system PhoPQ and its regulon (Groisman *et al.*, 1989; Miller *et al.*, 1989; Garvis *et al.*, 2001) and (2) the SPI-2 T3SS (Shea *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998). The intracellular proliferation defect of SPI-2 mutants was observed in primary macrophages and several macrophage-like cell lines as well as in epithelial cell lines (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Beuzón *et al.*, 2002). *S.* Typhimurium strains deficient in SPI-2 are highly attenuated in the murine systemic infection model (Shea *et al.*, 1996; Hensel *et al.*, 1998; Chan *et al.*, 2005).

The intracellular survival defect can be observed in most individual mutants, such as *sseB*, *ssaR* and *ssrB*. The *sseB* mutant of *S.* Typhimurium has a growth defect in murine macrophages (peritoneal, RAW264.7 and J774A.1), and in epithelial cells (HEp-2) but was not recovered from liver or spleen of infected BALB/c mice (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). SseB is a translocon constituent involved in pore formation in the host membrane for delivery of effector proteins. An *sseB* mutant does not secrete SseD and SseC, two other translocon proteins (Chakravortty *et al.*, 2005), and SseB is required for the translocation of effector proteins by intracellular *Salmonella* (Nikolaus *et al.*, 2001). Also, *ssaR* mutants of *S.* Typhimurium are defective in intracellular replication in murine macrophages (RAW264.7), are attenuated in BALB/c mice and are unable to secrete SPI-2 effectors (Shea *et al.*, 1996; Coombes *et al.*, 2004). Mutants of the two-component system SsrAB are unable to survive within murine macrophages (RAW264.7, J774A.1), are impaired in epithelial cell replication (HEp-2) and are avirulent in mice (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996).

Moreover, SPI-2 has also been shown to interfere with antigen presentation (Cheminay *et al.*, 2005; Tobar *et al.*, 2006; Halici *et al.*, 2008), cytokine production (Uchiya *et al.*, 2004) and cytotoxicity (Monack *et al.*, 2001; Paesold *et al.*, 2002). The critical role of the SPI-2 T3SS for *S.* Typhimurium is therefore well established. Although most SPI-2 genes were upregulated following *S.* Typhi uptake by human macrophages (Faucher *et al.*, 2006), the specific role of the SPI-2 T3SS of *S.* Typhi has not yet been reported. In this study, we targeted specific components of the secretion system, such as the translocon (*sseB*), the basal secretion apparatus (*ssaR*) and the regulator (*ssrB*) in order to investigate the contribution of the SPI-2 T3SS during interaction of *S.* Typhi with human macrophages.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown overnight statically (low aeration) in Luria–Bertani (LB) broth, unless indicated otherwise. When required, antibiotics or supplements were added at the following concentrations: 50 μg ampicillin ml^{-1}, 34 μg chloramphenicol ml^{-1}, 50 μg nalidixic acid ml^{-1} or 50 μg diaminopimelic acid ml^{-1}.

Construction of SPI-2 mutants. Deletion of *sseB*, *ssaR* and the entire T3SS of SPI-2 [Δ(*ssaU–ssrB*)] were generated by allelic exchange as described previously for the *ssrB* deletion (Faucher *et al.*, 2009) by using the overlap-extension PCR method (Basso *et al.*, 2002). Primers are listed in Table 2, and detailed information on the deletions is presented in Supplementary Fig. S2. Mutations were confirmed by PCR. All mutants have growth curves in LB broth similar to that of the wild-type strain (data not shown).

SseB antibodies and secretion assays. The amplified *sseB* gene of *S.* Typhi ISP1820 was cloned into pET-21a vector (pSIF128) and SseB proteins were purified by using Ni-nitrilotriacetic acid agarose beads (Qiagen) and polyclonal antibodies against SseB were raised in rabbits (GeneScript). For secretion assays, bacteria were grown in a low phosphate and magnesium medium (LPM; pH 5.8), corresponding to SPI-2-inducing conditions (Coombes *et al.*, 2004). Bacteria were pelleted, and supernatant was collected and precipitated as described previously (Faucher *et al.*, 2009). Samples were dissolved in an appropriate volume of SDS-PAGE loading buffer, according to the OD_{600} value of the original culture. Proteins were separated on 10 or 15 % (w/v) SDS-polyacrylamide gels and then transferred onto PVDF membranes and incubated with rabbit anti-GroEL (Sigma) or rabbit affinity-purified antibodies raised against recombinant SseB. Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used as the secondary antibody and was detected with the ECL plus Western blotting detection reagent (GE Healthcare).

Infection of human cultured macrophages. The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 (Wisent) containing 10 % (v/v) heat-inactivated fetal calf serum (Invitrogen), 25 mM HEPES (Wisent), 2 mM L-glutamine, 1 mM sodium pyruvate and 1 % modified Eagle's medium with non-essential amino acids (Wisent). A stock culture of these cells was maintained as monocyte-like, non-adherent cells at 37 °C in an atmosphere containing 5 % (v/v) CO_2. For macrophage infection, cells were seeded at 5×10^5 cells per well in 24-well tissue-culture dishes and were differentiated by addition of 10^{-7} M phorbol 12-myristate 13-acetate for 48 h. Macrophage infection was performed as described previously (Daigle *et al.*, 2001) unless specified otherwise. Briefly, bacteria were grown overnight without shaking in LB broth, usually reaching an OD_{600} of 0.6. Bacteria were added to the cell monolayer at an m.o.i. of 10 : 1, and were centrifuged for 5 min at 800***g*** to synchronize bacterial uptake. After incubation for 20 min at 37 °C, extracellular bacteria were removed by washing cells three times with prewarmed PBS, pH 7.4, and the infected monolayers were either lysed (T_0) or incubated for 2 h with medium containing 100 μg gentamicin ml^{-1} (Wisent) to kill extracellular bacteria, and then with 12 μg gentamicin ml^{-1} for the remainder of the experiment. Cells were lysed by addition of 1 ml 0.1 % (w/v) sodium deoxycholate in PBS per well, and the number of surviving bacteria was determined as c.f.u. by plating on LB agar. Survival (fold increase) was defined as the number of bacteria recovered 24 h after infection divided by the number of bacteria detected 2 h after infection. Results are expressed as the mean ± SEM of replicate experiments. The non-parametric Mann–Whitney test was used for statistical analysis. Where indicated, bacteria grown to stationary phase in LB broth were diluted in PBS or

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Characteristic	Reference or source
Strains		
***S.* Typhi**		
ISP1820	Wild-type *S.* Typhi	Hone *et al.* (1991)
DEF093	ISP1820 Δ*sseB*	This study
DEF148	ISP1820 Δ*ssaR*	This study
DEF149	ISP1820 Δ*ssrB*	Faucher *et al.* (2009)
χ8521	ISP1820 Δ*phoP*	R. Curtiss III, Arizona State University (ASU)
Ty2	Wild-type *S.* Typhi	R. Curtiss III, ASU
DEF473	Ty2 Δ*sseB*	This study
DEF566	ISP1820 Nal^r	This study
DEF574	ISP1820 Δ(*ssaU–ssrB*)	This study
χ4455	ISP1820 Δ*asd*	R. Curtiss III, ASU
DEF010	χ4455 gfp^+ (pHY11)	This study
DEF478	DEF010 Δ*sseB*	This study
***S.* Typhimurium**		
χ3339	Mouse-passaged isolate of SL1344 *rpsL hisG*	Gulig & Curtiss (1987)
DEF458	χ3339 Δ*sseB*	This study
Escherichia coli		
MGN-617	SM10 λpir *asd thi thr leu tonA lacY supE recA* RP4 2-Tc::Mu[λpir] Δ*asdA4*	Kaniga *et al.* (1998)
BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-), with DE3, a prophage carrying the T7 RNA *pol* gene	Novagen
Plasmids		
pET-21a	IPTG-inducible T7 RNA-polymerase-dependent expression vector, 6×His-Tag at C terminus, Ap^r	Novagen
pHY11	pYA810–*gfp*	H. Y. Kang, Pusan National University; Srinivasan *et al.* (1995)
pMEG-375	*sacRB mobRP4 ori*R6K. Cm^r Ap^r	R. Curtiss III, ASU
pSIF040	pMEG-375 with flanking region of *sseB* used for *sseB* deletion	This study
pSIF073	pMEG-375 with flanking region of *ssaR* used for *ssaR* deletion	This study
pSIF128	pET-21a with *sseB*	This study
pSIF187	pMEG-375 with flanking region of *ssaU* and *ssrB* used for entire SPI-2 deletion	This study

Table 2. Primers used in this study

Primer	Sequence (5′–3′)*
sseB-BF	CGGGATCCAATACGCACCACGGCTTTT
sseB-BR	*CCCCGGTG*GACCCTACAACAGGCACATGACAC
sseB-EF	*TTGTAGGGT*CCACCGGGGCTTGAGCATTAAG
sseB-ER	GCTCTAGAAATGGGACAGGCTCTTAGTTAGCAC
ssaR-BF	CGGGATCCTGCTGAGCGTCAACAGATTTATCC
ssaR-BR	*ATTCGTAAC*GTCCCCCGTTTTAAGTTGTCG
ssaR-EF	*ACGGGGGAC*GTTACGAATTGGAGAGCATGGTTG
ssaR-ER	GCTCTAGAAGCAGAAACCCCGCCATATC
sseBF-*Nde*I	GGAATTCCATATGTCTTCAGGAAACATCTTATG
sseBR-*Xho*I	CCGCTCGAGTGAATACGTTTTCTGCGCTATCA
ssaU-R	CGGGATCCAATACGCTATCTGGTGCTTG
ssaU-Fover	*TGTTTCGAC*TGCAGCCTTGTTACGTATGG
ssrB-Fover	*AAGGCTGCA*GTCGAAACACATCGGATGAAT
ssrB-R	GCTCTAGAAGGCGTAAGGCTCATCAAAAT

*Restriction enzyme sites are underlined. Letters in italics denote overlapping sequences.

opsonized for 20 min in 10 % normal human serum before being added to macrophages at an m.o.i of 10 : 1. Activation of macrophages was achieved by addition of 100 U gamma interferon (IFN-γ) ml^{-1} (PBL Biomedical Laboratories) in the medium 48 h prior to infection. The addition of IFN-γ was maintained throughout the infection.

For competitive index (CI) experiments in macrophages (Segura *et al.*, 2004), a spontaneous nalidixic acid-resistant (Nal^r) *S.* Typhi ISP1820 strain was used as the wild-type strain (DEF566). This Nal^r strain showed no intracellular attenuation compared with strain ISP1820 when both were used in a CI experiment in THP-1 macrophages (data not shown). The wild-type and mutant strains used for competition experiments were separately grown overnight (static) in LB broth, and a 1 : 1 mixture of the two cultures was added at an m.o.i. of 50 : 1 to the THP-1 cell monolayer, as described above. The numbers of viable intracellular bacteria were determined 2 and 24 h after infection by plating on LB agar and on LB agar with antibiotic. The CI for bacterial survival is defined as the mutant to wild-type ratio of bacteria recovered 24 h post-infection divided by the equivalent ratio of bacteria recovered 2 h post-infection. Statistical significance was evaluated by using Student's two-tailed *t*-test by comparing ratios (c.f.u. mutant/c.f.u. wild-type) at 24 and 2 h (Santiviago *et al.*, 2009).

Infection of human monocyte-derived macrophages (MDMs). MDMs were obtained by using previously published protocols (Baron-Bodo *et al.*, 2005; Romieu-Mourez *et al.*, 2006; Goubau *et al.*, 2009). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation from fresh apheresis with informed and institutionally approved consent forms from healthy donors. PBMCs were resuspended at 10^6 cells ml^{-1} in ISCOVE medium (Wisent) supplemented with 2 % human serum (Wisent), antibiotics and 700 U ml^{-1} recombinant human GM-CSF (a generous gift from Cangene Corporation). On day 7, adherent MDMs were harvested after three washes with PBS and gentle cell scraping and were identified based on assessment of CD14, CD64 and HLA-DR. MDMs were transferred to a 24-well tissue culture plate and further incubated for 3 days (to day 10). Infection of MDMs with *S.* Typhi was performed as described for THP-1 macrophages.

Microscopy of infected macrophages. THP-1 cells were seeded on to glass coverslips (12 mm diameter) in 24-well plates. The infection was then carried out using GFP-expressing strains. For fluorescence microscopy, cell monolayers were washed in PBS and then fixed in 4 % paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature. Coverslips were washed three times in PBS and mounted on Geltol (Immunon Thermo). Samples were analysed using a fluorescence microscope (Eclipse E600; Nikon).

RESULTS

SPI-2 mutants

S. Typhi ISP1820 isogenic strains harbouring mutations in genes encoding distinct components of the SPI-2 T3SS were constructed by allelic exchange (Supplementary Figs S1 and S2). Deletion of *sseB*, encoding a secreted protein, was selected to investigate the effect of a mutation in the translocon. Deletion of *ssaR* was used to target the basal apparatus component of the needle complex and deletion of *ssrB* was used to evaluate the effect of a mutation in the regulatory system of the SPI-2 T3SS. To confirm that the SPI-2 mutants (*ssaR* and *ssrB*) of *S.* Typhi were non-functional, detection of the SseB protein in the wild-type strain and SPI-2 mutants was monitored by using SPI-2-inducing conditions and anti-SseB antibodies. As expected, SseB was secreted in the culture supernatant of the wild-type strain, but not in the supernatant of the SPI-2 mutants (Fig. 1). In the bacterial pellet, the SseB protein was only detected in the wild-type and the *ssaR* mutant (Fig. 1). The *sseB* mutant was used as a control for the specificity of the antibody and GroEL was used as a control for bacterial cytoplasm leakage and was not detected in the culture supernatant. The data confirmed that the SPI-2 mutants were non-functional for secretion.

Phenotype of SPI-2 mutants within human macrophages

To evaluate bacterial survival within macrophages, THP-1 macrophages were infected with the wild-type strain and the isogenic SPI-2 mutants were grown at low aeration by using a gentamicin protection assay. The number of bacteria present at different times was determined by c.f.u. counts. Surprisingly, none of the specific deletion mutations in SPI-2-T3SS-encoding genes affected bacterial uptake or survival, as the number of bacteria recovered at different times after infection was similar to that of the wild-type strain (Fig. 2). As there were no differences observed between the different SPI-2 mutants, the *sseB* mutant was selected for further experiments.

The role of *S.* Typhi SPI-2 in survival in human macrophages was then assessed more specifically at different times after infection. *S.* Typhi had already begun to replicate 2 h after infection and continued for up to 3 days after infection (Fig. 3). A growth pattern similar to that for the wild-type strain was observed for the *sseB* mutant (Fig. 3). *S.* Typhi constitutively expressing green fluorescent protein (DEF010) and its *sseB* isogenic mutant (DEF478) were used to visualize bacteria within macrophages by fluorescence microscopy. Initially, many macrophages were infected with a single bacterium or a few bacteria (Fig. 3b). At 24 h post-infection, infected macrophages contained many bacterial clusters, suggesting that the bacteria had replicated (Fig. 3b). Interestingly, the

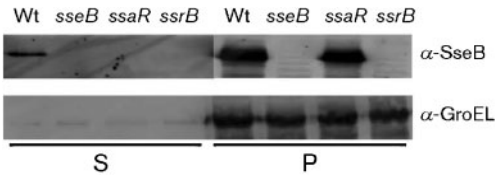

Fig. 1. *In vitro* secretion of SseB in SPI-2 mutants. Wild-type *S.* Typhi ISP1820 strain (Wt), and isogenic *sseB*, *ssaR* and *ssrB* deletion mutant strains were grown under SPI-2-inducing conditions (LPM; pH 5.8). Proteins in the supernatant (S) and bacterial pellet (P) were harvested and examined by Western blotting to detect SseB and GroEL.

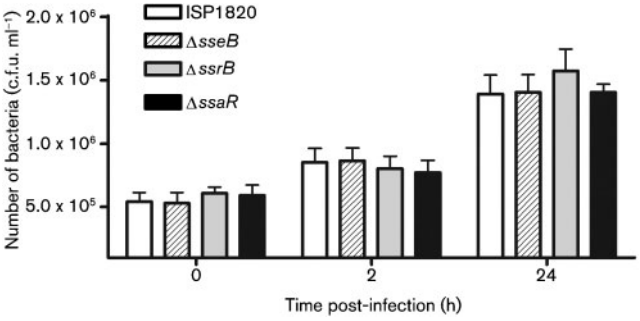

Fig. 2. Effect of mutations in SPI-2 during interactions with human macrophages. THP-1 macrophages were infected with wild-type (ISP1820), *sseB*, *ssaR* or *ssrB* deletion mutant strains and the number of intracellular bacteria were determined at different times after infection. Data presented are the mean ± SEM of at least three independent experiments performed in duplicate.

infected cell monolayer was mainly intact, even 96 h post-infection (Fig. 3b) and only a few cells were detached.

To evaluate the usefulness of our infection model, an isogenic strain carrying a *phoP* deletion, a global transcriptional activator involved in *Salmonella* virulence and intracellular survival within macrophages (Miller *et al.*, 1989), was used as a control. The isogenic *phoP* mutant showed significantly reduced survival (Fig. 4), suggesting that the THP-1 macrophages are efficient for bacterial killing.

We next investigated if the absence of survival defects for an SPI-2 mutant was unique to the *S.* Typhi strain ISP1820. An *sseB* deletion was generated in *S.* Typhi strain Ty2 and no significant difference in intracellular survival between the wild-type strain and the SPI-2 mutant was observed (Fig. 4). As a positive control, the role of SPI-2 of *S.* Typhimurium in human macrophages was then investigated. An *sseB* mutant of *S.* Typhimurium strain SL1344 was constructed and its contribution to survival in macrophages was evaluated. The *sseB* deletion in *S.* Typhimurium significantly reduced intracellular survival of the SPI-2 mutant in THP-1 macrophages (Fig. 4).

Influence of growth conditions

Salmonella can enter macrophages by several endocytic processes, which can be affected by the bacterial growth conditions prior to infection. Bacteria were grown to late exponential phase under low aeration (static), and were grown to stationary phase with or without opsonization. Regardless of the growth conditions prior to infection, there was no difference between the wild-type and the *sseB* mutant (Fig. 5). The uptake level of bacteria grown in different conditions was also similar. The number of bacteria recovered after 24 h of infection was always higher than the number of bacteria recovered after 2 h (fold increase >1) when bacteria were grown to late exponential phase without agitation (Figs 2–5). However, when *S.* Typhi uptake was investigated by using bacteria grown with shaking to stationary phase, with or without opsonization, a constant number of bacteria between 2 and 24 h after infection (fold increase of $\cong 1$) was observed (Fig. 5).

Activation of macrophages

IFN-γ is a critical cytokine that plays a central role in host defence against intracellular pathogens, including *Salmonella*

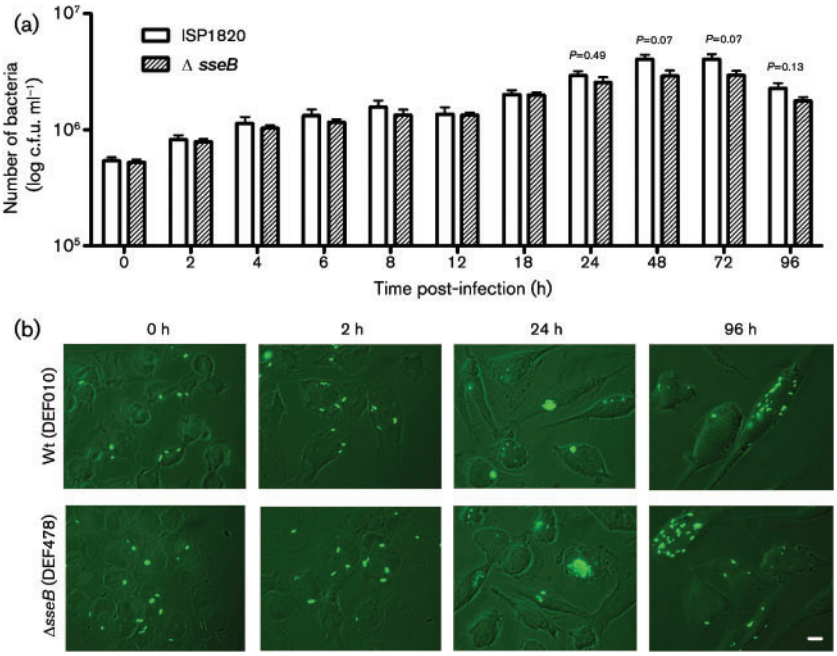

Fig. 3. Time course of *S.* Typhi survival in human macrophages. THP-1 macrophages were infected with *S.* Typhi ISP1820 and its isogenic *sseB* mutant. (a) The number of intracellular bacteria were determined at different times after infection. Data presented are the mean ± SEM of three independent experiments performed in duplicate. *P*-values are indicated for late time points. (b) Fluorescence microscopy of fixed macrophages at different times after infection with bacteria constitutively expressing the green fluorescent protein (original magnification, ×1000).

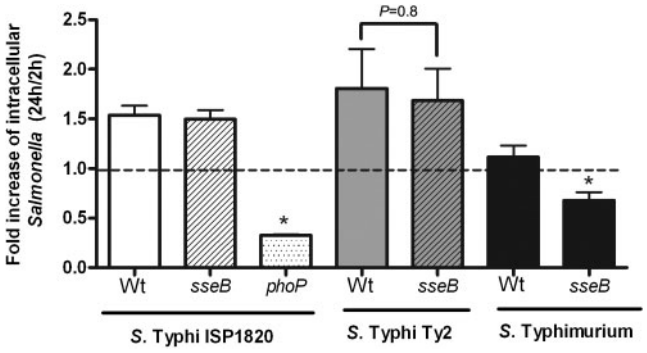

Fig. 4. Role of SPI-2 in intracellular survival in different strains of *Salmonella*. THP-1 macrophages were infected with *S.* Typhi ISP1820 (Wt) and its isogenic *sseB* and *phoP* mutants, *S.* Typhi strain Ty2 and its isogenic *sseB* mutant, and *S.* Typhimurium SL1344 and its isogenic *sseB* mutant. Intracellular survival was determined by comparing the number of bacteria 24 h post-infection with the number of bacteria at 2 h (fold increase). Data presented are the mean ± SEM of at least three independent experiments performed in duplicate. Mutants demonstrating a significant ($P<0.05$) difference compared with the isogenic wild-type strain are indicated (*).

(Muotiala & Mäkelä, 1993; Rosenberg *et al.*, 2002). As *Salmonella* may encounter activated macrophages during its life cycle inside the host, we investigated the role of SPI-2 during infection of activated macrophages. THP-1 macrophages were primed with IFN-γ for 48 h and then infected with wild-type *S.* Typhi and its isogenic *sseB* SPI-2 mutant. Following IFN-γ treatment, a small number of bacteria were recovered from macrophages (Fig. 5), although there was no significant difference between the wild-type and the SPI-2 mutant in the IFN-γ-primed macrophages.

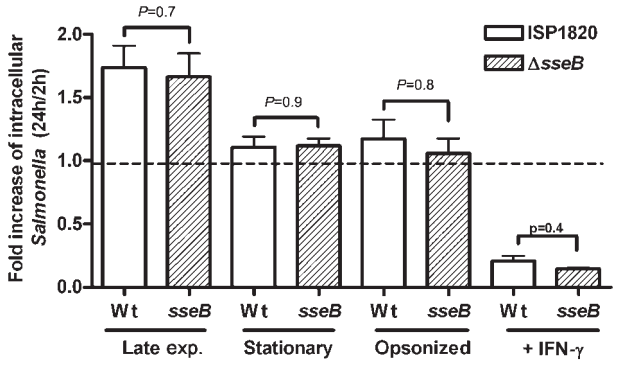

Fig. 5. Effect of growth conditions, opsonization or macrophage activation on intracellular survival of *S.* Typhi. THP-1 macrophages were infected with *S.* Typhi ISP1820 (Wt) and its isogenic *sseB* mutant grown in LB: cultures were overnight static (late exponential growth), overnight shaking (stationary phase) with or without opsonization with human serum (opsonized), or by using activated macrophages primed with IFN-γ. Intracellular survival was determined by comparing the number of bacteria 24 h post-infection with the number of bacteria at 2 h (fold increase). Data presented are the mean ± SEM of at least three independent experiments performed in duplicate. Exact *P*-values are also indicated.

Mixed infection assay of human macrophages

Survival of the wild-type strain and its isogenic mutants was evaluated simultaneously by using the CI assay. The CIs for the *sseB* and *phoP* mutants within THP-1 cells after mixed infections with the wild-type strain were 1.19 ($P=0.50$) and 0.27 ($P=0.0048$), respectively. These results are in agreement with our data from individual strains during infection and confirm that SPI-2 does not affect survival in human macrophages and that the PhoPQ regulon is of importance.

Infection of human MDMs

It has been shown that macrophages from immortalized cell lines allow moderate growth of an SPI-2 mutant of *S.* Typhimurium compared with a greater level of growth defect in primary cells (Hensel *et al.*, 1998; Helaine *et al.*, 2010). Human MDMs were infected with the wild-type *S.* Typhi strain and the isogenic *sseB* mutant. The number of bacteria recovered decreased over time, suggesting a defect in survival; however, the number of recovered bacteria was similar for both the wild-type strain and the SPI-2 isogenic mutant (Fig. 6).

Complete deletion of the SPI-2 T3SS

To validate and understand the surprising results obtained with the different SPI-2 mutants, a 25 kb deletion of the entire T3SS (*ssaU–ssrB*) was generated and tested for survival in THP-1 macrophages. Deletion of the T3SS of SPI-2 did not affect bacterial uptake or survival, as the

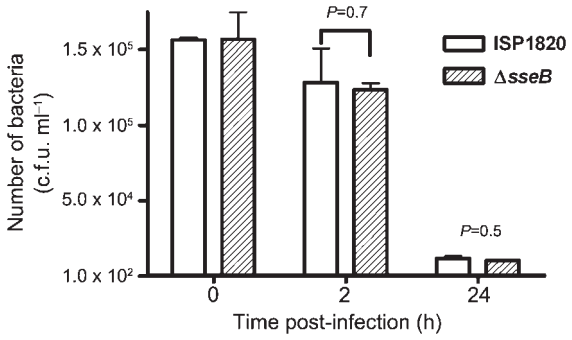

Fig. 6. Role of *S.* Typhi SPI-2 in human MDMs. Cells were infected with *S.* Typhi ISP1820 and its isogenic *sseB* mutant and intracellular bacteria were determined at different times after infection. Representative mean ± SEM of a duplicate MDM experiment from a single donor are shown. *P*-values are indicated.

number of bacteria recovered at different times after infection was similar to that of the wild-type strain (Fig. 7).

DISCUSSION

As the ability to survive and replicate inside macrophages is tightly associated with the *S.* Typhimurium SPI-2 T3SS and with virulence (Fields *et al.*, 1986), and since very little information regarding the involvement of *S.* Typhi SPI-2 T3SS in this process is available, the role of the *S.* Typhi SPI-2 T3SS inside human macrophages was investigated. Mutants corresponding to specific components of the secretion system were tested for intracellular survival, including a translocon mutant (*sseB*), an apparatus mutant (*ssaR*) and a transcriptional regulator mutant (*ssrB*), the last of these being required for expression of SPI-2 genes. These SPI-2 mutants were previously shown to be defective in intracellular replication of *S.* Typhimurium (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). Moreover, any mutations in SseB lead to structural alterations that abolish translocon function, resulting in replication defects in macrophages (Hölzer & Hensel, 2010). SseB was not secreted in the *ssaR* and *ssrB* mutants, confirming that the SPI-2 T3SS is not functional (Fig. 1). Surprisingly, mutations in either *sseB* (translocon), *ssaR* (basal component) or *ssrB* (regulator), all of which abrogate SPI-2 T3SS function or expression, did not affect *S.* Typhi uptake and survival in human cells. Similar results were obtained during competition assays, when the entire SPI-2 T3SS (Δ*ssaU–ssrB*) was deleted (Fig. 7), or when macrophages derived from human monocytic cells were used (Fig. 6), confirming that intracellular survival of *S.* Typhi inside human macrophages is independent of the SPI-2 T3SS. The number of intracellular bacteria corresponding to the *sseB* SPI-2 mutant increased inside macrophages with a pattern similar to that of the wild-type strain (Fig. 3). Despite not demonstrating a key role for intracellular survival, the SPI-2 genes of *S.* Typhi are highly induced inside human macrophages (Faucher *et al.*, 2006), suggesting that the difference may not be at the transcriptional level.

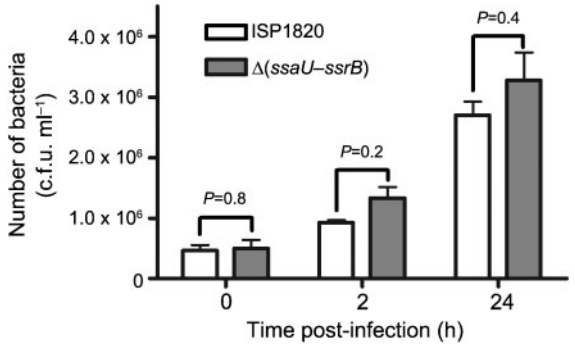

Fig. 7. Effect of complete deletion of the SPI-2 T3SS locus in *S.* Typhi during interactions with human macrophages. THP-1 macrophages were infected with the wild-type (ISP1820) and its isogenic mutant harbouring a complete deletion of the SPI-2 T3SS (*ssaU–ssrB*) and intracellular bacteria were determined at different times after infection. Data presented are the mean ± SEM of three independent experiments performed in duplicate. *P*-values are indicated.

Moreover, no defect in survival was observed for an *sseB* SPI-2 mutant of *S.* Typhi strain Ty2, suggesting that the absence of a phenotype for an SPI-2 mutation in *S.* Typhi was not strain-specific (Fig. 4). However, a *phoP* mutant of *S.* Typhi known to be involved in intracellular replication of *S.* Typhimurium (Groisman *et al.*, 1989; Miller *et al.*, 1989) was defective in this model, as was an *sseB* mutant of *S.* Typhimurium (Fig. 4).

As bacterial growth conditions and opsonization affect uptake and have a significant influence on bacterial survival, replication and virulence gene expression in *S.* Typhimurium (Hensel *et al.*, 1997, 1998; Drecktrah *et al.*, 2006), we investigated the influence of growth condition on *S.* Typhi survival in macrophages. Growth to late exponential phase under low aeration promotes SPI-1 expression and T3SS-mediated invasion of *S.* Typhimurium (Lee *et al.*, 1992), while aerated stationary phase bacterial cultures or opsonization promote phagocytosis and low cytotoxicity (Hensel *et al.*, 1998; Lundberg *et al.*, 1999; Drecktrah *et al.*, 2006). Aerated stationary phase or opsonized bacteria are then usually used to study the effect of SPI-2 mutants, as the replication defect of *S.* Typhimurium is overemphasized (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; see also Fig. 5). Under all of these conditions, *S.* Typhi survived and no differences were observed between the wild-type strain and the *sseB* SPI-2 mutant (Fig. 5). Moreover, there was no significant difference observed in the uptake levels of *S.* Typhi between the different growth conditions or with or without opsonization. The presence of the Vi capsule, specific to *S.* Typhi, may be responsible for the lack of difference in bacterial uptake under different growth conditions. Interestingly, an increase in the number of bacteria recovered 24 h post-infection (ratio at 24 h : 2 h >1) was only observed when *S.* Typhi was grown to late exponential phase. It will be interesting to investigate further the mechanisms of entry in macrophages of *S.* Typhi, as they may be related to its survival.

Activated macrophages play a central role in host defence and have been shown to increase phagocytic and killing activities. Survival of *S.* Typhi in human macrophages primed with IFN-γ was greatly reduced, but no significant difference was observed between the wild-type and the SPI-2 mutant. Similarly, no differences between the wild-type and the SPI-2 mutant were observed during survival in whole human blood (data not shown) or in invasion and survival of human epithelial cells (data not shown). It is possible that SPI-2 effectors may be translocated by the SPI-1 T3SS when the SPI-2 T3SS is mutated, but a double SPI-1 (*invA*)/SPI-2 (*ssaR*) mutant in *S.* Typhi survives and replicates to a level similar to that of the isogenic wild-type strain in THP-1 macrophages (data not shown). Thus, in

contrast with *S.* Typhimurium, the SPI-2 T3SS of *S.* Typhi is not critical for intracellular survival in human macrophages.

S. Typhimurium and *S.* Typhi are closely related serovars, sharing 90 % of their DNA (McClelland *et al.*, 2001). There are no major genomic differences between the SPI-2 T3SS of *S.* Typhimurium and *S.* Typhi that may explain these phenotypic differences (Sabbagh *et al.*, 2010). Phylogenetic analyses suggest a probable difference in functionality between these serovars, as variations in translocon genes *sseC* and *sseD* and in effectors *sseF* and *sifA* were observed (Eswarappa *et al.*, 2008). The major difference between these serovars seems to be in the diversity of the effector contents, which may contribute to host adaptation. Many SPI-2 effectors characterized in *S.* Typhimurium are missing in *S.* Typhi (*sseI*, *gogB*, *spvB*, *spvC*, *sseK1*, *sseK2* and *sseK3*) or are pseudogenes (*sopD2* and *sseJ*). Several of these effectors mediate long-term systemic infection in mice (Lawley *et al.*, 2006; McLaughlin *et al.*, 2009; Ruiz-Albert *et al.*, 2002). As some effectors are not present in *S.* Typhi, this emphasizes that there are differences in the virulence mechanisms responsible for systemic disease caused by *S.* Typhimurium in susceptible mice compared with those dictating systemic disease caused by *S.* Typhi in humans. The majority of the studies on maturation and trafficking of *S.* Typhimurium effectors were performed with epithelial cells, and when macrophages were used, they were usually of murine origin. Thus, it is premature to determine or hypothesise whether the absence of a survival defect is related to a difference in the cell type and/or the host source or to a genetic difference between *S.* Typhi and *S.* Typhimurium.

It is thus possible that the host plays an essential role and may be able to discriminate between these serovars. Indeed, mutations in homologous genes that attenuate *S.* Typhimurium in mice have given different results for *S.* Typhi when administered to humans as a potential vaccine (Garmory *et al.*, 2002). Moreover, differences in survival in human macrophages between the two serovars were previously observed, with a higher rate of survival for *S.* Typhi (Alpuche-Aranda *et al.*, 1994; Ishibashi & Arai, 1995; Schwan *et al.*, 2000), similar to what we observed here. This may be because *S.* Typhi is less cytotoxic for human cells (Schwan *et al.*, 2000; Fig. 3 and data not shown). *S.* Typhi increases rapidly and no lag phase is observed in THP-1 macrophages (Figs 2, 3 and 7). Differential survival between these serovars was also detected in susceptible mice, where *S.* Typhi was rapidly eliminated (Xu *et al.*, 2009). The use of *S.* Typhimurium in mice as a surrogate for *S.* Typhi in humans has many real advantages but may also be misleading.

As SPI-2 is present in *S.* Typhi, it is unlikely that there is no evolutionary advantage in carrying it. It could be used during infection of other cell types, such as dendritic or natural killer cells. The SPI-2 T3SS may be critical when the bacteria are in a specific human niche with high immunological cell content, such as the spleen. The SPI-2 T3SS may be required to modulate the host immune system to establish long-term asymptomatic infection, which will not necessarily translate into defects in intracellular survival. Further investigation is needed to clarify the role of the *S.* Typhi SPI-2 T3SS in human cells.

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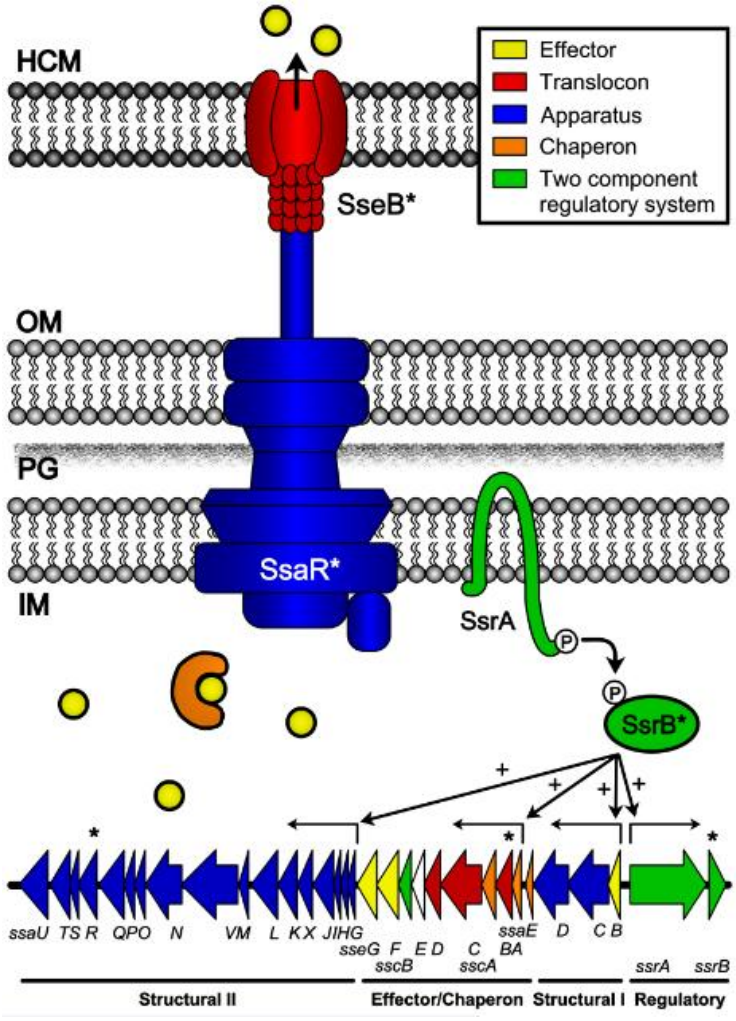
Figures supplémentaires de l'article S1. Chantal G. F., Ferraro, E., Sabbagh, S. C. and F. Daigle. 2010. Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is independent of *Salmonella* pathogenicity island (SPI)-2 independent. Microbiology, **156:**3689-3698. (Co-première auteure)

Figure S1. *Salmonella* pathogenicity island-2 type three secretion system.

Model of the SPI-2 type three secretion system including the localization of the proteins (*) and their corresponding genes that were used to generate deletion mutants used in this study. HCM stands for host cell membrane, OM for outer membrane, PG for peptidoglycan, IM for inner membrane. Adapted from (128).



IV

Figure S2 : Detailed description of mutants generated during this study

1) *sseB* deletion resulting in strain DEF093

Primers used :

sseB BF cgggatccAATACGCACCACGGCTTTTAAC
sseB BR ccccggtggACCCTACAACAGGCACATGACAC
sseB EF ttgtagggtCCACCGGGGCTTGAGCATTAAG
sseB ER gctctagaAATGGGACAGGCTCTTAGTTAGCAC

>STY1722 *sseB* (*Salmonella enterica* subsp. enterica serovar Typhi str. CT18) plus 2000 bp. upstream

atatcatcaacattatttattatcgttaccataacagcgcacttgtagtgagtgagcaagagtaaagtaaaaatatcttagagcctatcccaccaggcgttaattggcgcagccagtttggacacggatagcgc
gcaaaaaccggagcgtacacgtagtacgtgaggattttgagcactgcccaggttcaaaatggcaagtaaaatagccctaatgggacaggctcttagttagcacgttaattatctatcgtgtatatggaggg
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cagagagccccaaaaagagcagagagacagagagtattcttcataacctatttccacaaggcgttgccggggttaaccaggaggccgagaaggatttaaagaaaatagtaagtttgtttaaacaacttga
agtacgactgaaacaacttaatgctcaagccccggtggagataccgtcaggaaaaacaaaaaggtaaagcataATGTCTTCAGGAAACATCTTATGGGGAAGTCAAA
ACCCTATTGTGTTTAAAAATAGCTTCGGCGTCAGCAACGCTGATACCGGGAGCCAGGATGACTTATCCCAGCAAAATCCGT
TTGCCGAAGGGTATGGTGTTTTGCTTATTCTCCTTATGGTTATTCAGGCTATCGCAAATAATAAATTTATTGAAGTCCAGAA
GAACGCTGAACGTGCCAGAAATACCCAGGAAAAGTCAAATGAGATGGATGAGGTGATTGCTAAAGCAGCCAAAGGGGAT
GCTAAAACCAAAGAGGAGGTGCCTGAGGATGTAATTAAATACATGCGTGATAATGGTATTCTCATCGATGGTATGACCAT
TGATGATTATATGGCTAAATATGGCGATCATGGGAAGCTGGATAAAGGTGGCCTACAGGCGATCAAAGCGGCTTTGGATA
ATGACGCCAACCGGAATACCGATCTTATGAGTCAAGGGCAGATAACAATTCAAAAAATGTCTCAGGAGCTTAACGCTGTC
CTTACCCAACTGACGGGGCTTATCAGTAAGTGGGGGGAAATTTCCAGTATGATAGCGCAGAAAACGTATTCATGAaaaaagac
ccgaccctacaacaggcacatgacacgatgcgatttttccggcgtggcggctcgctgcgtatgttgttggatgacgatgttacacagccgcttaatactgtgtatcgctatgccatgcagcttatggaggta
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gacgcgcggcacaaattaagattgatgcgccgcaggcgccatgggccgcagcggaatgctatctcgcgtgtgataacgtctgttatgcaataaaagcgttaaaagccgtggtgcgtatttgcggcgagg
tcagtgaacatcaaatcctccgactacgtgcagaaaagatgttacagcagctttctgacaggagctaaaaatgaatcgaattcacagtaatagcgatagcgccgcaggagtaaccgccttaacacatcatc
acttaagcaatgtcagttgcgtttcctcgggttcgctgggaaagcgccagcatcgtgtgaattctac

591 bp were deleted from *sseB*; 35 bp upstream and 11 bp downstream were also deleted.

2) *ssaR* deletion resulting in strain DEF148

Primers used :

ssaR BF cgggatccTGCTGAGCGTCAACAGATTTATCC

ssaR BR attcgtaacGTCCCCCGTTTTAAGTTGTCG

ssaR EF acgggggacGTTACGAATTGGAGAGCATGGTTG

ssaR ER gctctagaAGCAGAAACCCCGCCATATC

>STY1701 *ssaR* (*Salmonella enterica* subsp. enterica serovar Typhi str. CT18) plus 600 bp. upstream

tttctgctgagcgtcaacagatttatcctgctcctcctgtggtagtccctgtatatttaggctggtgtcagcttacattaattgaacttgagtctatcgaaatcggcatgggcgttcggattcattgctttggcgaca
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gttaaagagtgatggaacgtcttcagtcgaacttgagcagataccacacaacaggtgctctttgagattggacgtgcgagtctggaaattggacaattacgacaacttaaaacgggggacgttttgcctgtag
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tgataagaaaaatataatatgcgaacaatataatagcgttccaggtcgtgtcatgagagatacagtATGTCTTTACCCGATTCGCCTTTGCAACTGATTGGTATATTGT
TTCTGCTTTCAATACTGCCTCTCATTATCGTCATGGGAACCTCTTTCCTTAAACTGGCGGTGGTATTTTCGATTTTACGAAAT
GCTCTGGGTATTCAACAAGTTCCTCCAAATATCGCACTGTATGGCTTCGCGCTTGTACTTTCCTTATTCATTATGGGGCCGA
CGCTATTAGCTGTAAAAGAGCGCTGGCATCCGGTTCAGGTCGCTGGCGCTCCTTTCTGGACGTCTGAGTGGGACAGTAAAG
CATTAGCGCCTTATCGACAGTTTTTGCAAAAAAACTCTGAAGAGAAGGAAGCCAATTATTTTCGGAATTTGATAAAACGA
ACCTGGCCTGAAGACATAAAAAGAAAGATAAAACCTGATTCTTTGCTCATATTAATTCCGGCATTTACGGTGAGTCAGTTA
ACGCAGGCATTTCGGATTGGATTACTTATTTATCTTCCCTTTCTGGCTATTGACCTGCTTATTTCAAATATACTGCTGGCTAT
GGGGATGATGATGGTATCGCCAATGACCATTTCATTACCGTTTAAGCTGCTAATATTTTTACTGGCAGGCGGTTGGGATCT
GACACTGGCGCAATTGGTACAGAGCTTTTCATGAatgattctgaattgacgcaatttataacgcaacttttatggatcgtccttttacgtctatgccggtggtgttggtggcat
cggtagttggtgtcatcgtaagtcttgtccaggccttgactcaaattcaggaccaaacgctacagttcatgattaaattattagcaattgcaataactttgatggtcagctacccatggcttagcggaatcctgtt
gaattatacccggcagataatgttacgaattggagagcatggttgaatgacacaacaggtaaatgagtggcttattgcattggctgtggcttttattcgaccattgagccttttctttattacttcctctattaaaaa
gtggcagtttaggggccgcgcttttacgtaatggcgtgcttatgtcacttacctttcccatattaccaatcatttaccagcagaagattatgatgcatattggtaaagattacagctggttagggttagtcaccgg
agaggtgattattggtttttaattgggttttgtgcggcggttccctttgggccgttgatatggcggggtttctgcttgatactttacgtggcgcgacaatgggtacga

648 bp were deleted from *ssaR*; 211 bp upstream and 238 bp downstream were also deleted.

VI

3) *ssrB* deletion resulting in strain DEF149

Primers used :

ssrB BF cgggatccAAGGCTGTTTAGGTCAAATAGGGC

ssrB BR cttcgggcgGATAAGTATGTCAGGCTCGTATGCG

ssrB EF atacttatcCGCCCGAAGAATGAGGTTAATAG

ssrB ER gctctagaTCGCCGATAGAATACGACATGG

>STY1729 *ssrB* (*Salmonella enterica* subsp. enterica serovar Typhi str. CT18) plus 600 bp. upstream

gattcttttggttgatgatgccgatattaatcgggatatcatcggcaaaatgcttgtcagcctgggccaacacgtcactattgccgccagtagtaacgaggctctgactttatcacaacagcaacgattcgattt
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AACGGCATTATGAATGCCTTATTACCCTGGCCTCATTTTAAAATTGTAGAGCATGTTAAAAATGGTCTTGAGGTTTATAAT
GCCTGTTGCGCATACGAGCCTGACATACTTATCCTTGATCTTAGCTTACCTGGCATCAATGGCCTGGATATCATTCCTCAAT
TACATCAGCGTTGGCCAGCAATGAATATTCTGGTTTACACAGCATACCAACAAGAGTATATGACCATTAAAACTTTAGCCG
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cacgctaagggcatggcagcgacgctatggtttattgaaaccgcagcgtactgatggcgggcatcgcttatacagcgatgacgatattcgacaagcgcttagcatcctcgactgggtgagaaaaggcgt
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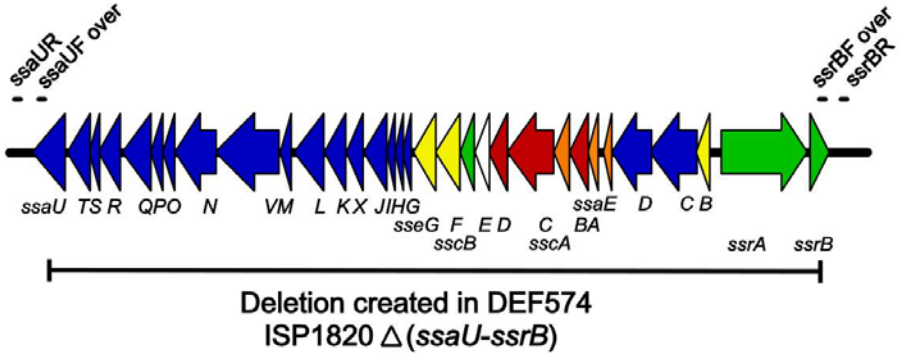
446 bp were deleted from *ssrB*.

4) Entire SPI-2 deletion resulting in strain DEF574



Primers used :

ssaU R cgggatccAATACGCTATCTGGTGCTTG
ssaU F over tgtttcgacTGCAGCCTTGTTACGTATGG
ssrB F over aaggctgcaGTCGAAACACATCGGATGAAT
ssrB R gctctagaAGGCGTAAGGCTCATCAAAAT

>STY1698 *ssaU* (*Salmonella enterica* subsp. enterica serovar Typhi str. CT18) plus 600 bp. upstream

tgggaccggcaggcttctggatggggttcatcatcggcctgacctcggcggcggtgttaatgatgctacggatgcgctatctacaacgtcagccgtccgccatcattttgcagcgcgctgcgcgataata
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CATAAGAAAATGGTTTATTTACTAATTGTAATGTGAACGTTATTGACTCAATCAGTATCAAAATCATCTTTTCAGTAAAGA
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atcataatc

VIII

>STY1729 *ssrB* (*Salmonella enterica* subsp. enterica serovar Typhi str. CT18) plus 600 bp. upstream

cgattcgatttagtactgattgacattagaatgccagaaatagatggtattgaatgtgtacaattatggcacgatgagccgaataatttagatcctgactgcatgtttgtggcgctatccgctagcgtagcgaca
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CATCATTAACGGCATTATGAATGCCTTATTACCCTGGCCTCATTTTAAAATTGTAGAGCATGTTAAAAATGGTCTTGAGGTT
TATAATGCCTGTTGCGCATACGAGCCTGACATACTTATCCTTGATCTTAGCTTACCTGGCATCAATGGCCTGGATATCATTC
CTCAATTACATCAGCGTTGGCCAGCAATGAATATTCTGGTTTACACAGCATACCAACAAGAGTATATGACCATTAAAACTT
TAGCCGCAGGTGCTAATGGCTATGTTTTAAAAAGCAGTAGTCAGCAAGTCCTGTTAGCGGCATTGCAAACAGTCGCAGTA
AACAAGCGTTACATTGACCCGACGTTGAATCGGGAAGCTATCCTGGCTGAATTAAACGCTGACACGACCAATCATCAACT
GCTTACTTTGCGCGAGCGTCAGGTTCTTAAACTTATTGACGAGGGGTATACCAATCATGGGATCAGCGAAAAGCTACATAT
CAGTATAAAAACCGTCGAAACACATCGGATGAATATGATGAGAAAGCTACAGGTTCATAAAGTGACAGAGTTACTTAACT
GCGCCCGAAGAATGAGGTTAATAGAGTATTAAccaggggcatccgatggtattaagcattggtcatatttgatgagcctacgccacgcagtattgctcatcatcgacaaa
atccatacggatgccctggtatgccgcaccatttatcactaccttagtcttcatttaatcatgatatagtagaatccccttatttaacgggctttaccatgtcgtattctatcggcgaatttgccagactatgcggta
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aaaaggcgtaccgataagtcaggtcaaacccttactgtcgcgtccggtcattcgcctgggcgataactggataacgattcaggagacgatgcttcaacatctccacgaagggcgaattgacgcgctgcg
gcagttgatttatgactgtggccgggaatatccccgcgcagaactggtgacccatttattgcgtccgttgcgcagcaaagtgtccgcgcatcttcccgccgtgatgacgctgcgcgaaatatt

61 bp of *ssaU* and 147 bp of *ssrB* are still in Typhi's chromosome for DEF574, resulting in a deletion of exactly 25,053 kb.

MINIREVIEW

So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi

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Abstract

Salmonella enterica represents a major human and animal pathogen. Many *S. enterica* genomes have been completed and many more genome sequencing projects are underway, constituting an excellent resource for comparative genome analysis studies leading to a better understanding of bacterial evolution and pathogenesis. *Salmonella enterica* serovar Typhimurium and Typhi are the best-characterized serovars, with the first being involved in localized gastroenteritis in many hosts and the latter causing a systemic human-specific disease. Here, we summarize the major genetic differences between the two different serovars. We detail the divergent repertoires of the virulence factors responsible for the pathogenesis of the organisms and that ultimately result in the distinct clinical outcomes of infection. This comparative genomic overview highlights hypotheses for future investigations on *S. enterica* pathogenesis and the basis of host specificity.

Introduction

Salmonella evolved as an intracellular pathogen after diverging from a common ancestor with *Escherichia* 100–150 million years ago (Doolittle *et al.*, 1996). The nomenclature and taxonomy of *Salmonella* are complex, controversial, have changed over the years and are still evolving. The genus *Salmonella* is composed of two distinct species: *Salmonella bongori*, a commensal of cold-blooded animals, and *Salmonella enterica* (divided into six subspecies) (Le Minor *et al.*, 1987; Reeves *et al.*, 1989). The subspecies are classified into over 50 serogroups based on the O (somatic) antigen, and divided into >2400 serovars based on the H (flagellar) antigen. Some serovars are ubiquitous and generalists, while others are specifically adapted to a particular host. Only a small fraction of serovars are associated with human infections and the majority belong to *S. enterica* ssp. I. *Salmonella enterica* ssp. I is responsible for two types of disease in humans due to ingestion of contaminated food or water: gastroenteritis, a localized infection or enteric fever (typhoid), a severe systemic infection.

Gastroenteritis is caused mainly by *S. enterica* serovar Typhimurium (*S.* Typhimurium) and *S.* Enteritidis. *Salmonella enterica* serovar Typhimurium can colonize and infect a broad spectrum of warm- and cold-blooded hosts, belongs to serogroup B and is a prototroph (Fig. 1). Typhoid fever, a life-threatening illness that remains a global health problem, is caused mainly by *S. enterica* serovar Typhi (*S.* Typhi), and a clinically indistinguishable condition is caused by *S.* Paratyphi A. *Salmonella enterica* serovar Typhi is a host-restricted serovar that specifically infects humans, belongs to serogroup D and is an auxotroph (Fig. 1). As *S.* Typhi is restricted to humans, there are no suitable animal models. In order to study typhoid fever pathogenesis, *S.* Typhimurium has been used for many years in a systemic infection model using susceptible mouse strains harbouring a mutation in the Nramp1 (Slc11a1) protein (Vidal *et al.*, 1995). Moreover, the use of *S.* Typhimurium with strains of mice that possess the Nramp$^{+/+}$ allele, which are consequently resistant to the infection, represents a model mimicking the long-term persistence observed in *S.* Typhi carriers (Monack *et al.*, 2004). These models have been crucial in

FEMS MICROBIOLOGY LETTERS

understanding systemic infections by *S. enterica*. However, as each serovar causes a distinct type of disease in humans, conclusions regarding *S.* Typhi pathogenesis in humans must be interpreted carefully.

Many recent reviews have extensively covered the literature on the pathogenesis of *S. enterica* (Grassl & Finlay, 2008; Haraga *et al.*, 2008; Tsolis *et al.*, 2008; McGhie *et al.*, 2009). This review presents a comparative analysis of the major genetic differences between *S.* Typhimurium and *S.* Typhi and how this may contribute to our understanding of typhoid pathogenesis.

Genetic diversity

Organization of genomes allows us to gain a better understanding of the mechanisms by which species or serovars have evolved. Analysis of the chromosomal gene arrangement revealed that the genomic backbone of *S.* Typhimurium is very similar to the *Escherichia coli* genome. However, major differences in gene order have been observed in the *S.* Typhi chromosome. Differences in the *S.* Typhi genome occur mainly because of genomic rearrangements involving recombination between different rRNA operons (Liu & Sanderson, 1995; Liu & Sanderson, 1996) or IS*200* elements (Alokam *et al.*, 2002). Each serovar evolves through the acquisition of genetic elements by horizontal gene transfer or by gene degradation. The genomes of *S.* Typhimurium strain LT2 and *S.* Typhi strain CT18 are composed of 4 857 432 and 4 809 037 bp, respectively (Fig. 2) (McClelland *et al.*, 2001; Parkhill *et al.*, 2001). Both serovars share about 89% of genes (McClelland *et al.*, 2001). Differences between *S.* Typhimurium and *S.* Typhi include $\approx$ 480 genes unique to *S.* Typhimurium and $\approx$ 600 genes unique to *S.* Typhi (Parkhill *et al.*, 2001). *Salmonella* pathogenicity islands (SPIs), plasmids, functional prophages and phage remnants contribute significantly to the genetic diversity among *S. enterica* strains (Rotger & Casadesús, 1999; Boyd & Brüssow, 2002) and will be discussed below.

The low level of genetic variation observed in *S.* Typhi genomes of distinct isolates from around the world revealed a highly conserved and clonal relation, suggesting that they emerged from a single progenitor, making *S.* Typhi a monomorphic organism (Baker & Dougan, 2007; Holt *et al.*, 2008). Clonality is often encountered in human-restricted pathogens (Achtman, 2008). There is very little evidence of adaptive selection in *S.* Typhi genes, with the exception of a recent evolution in phenotypic traits that includes the acquisition of resistance to fluoroquinolones (Chau *et al.*, 2007; Le *et al.*, 2007). Examination of DNA sequences and the rate of change of single-nucleotide polymorphisms suggest that *S.* Typhi may be only 50 000 years old, a short time frame for bacteria to accumulate diversity (Selander *et al.*, 1990; Kidgell *et al.*, 2002a, b; Roumagnac *et al.*, 2006). This situation strongly suggests that evolution in the *S.* Typhi strain population is mainly characterized by loss of gene function. *Salmonella enterica* serovar Typhi is an example of reductive evolution, where the adaptation to its human niche has led to the functional inactivation of genes, due to certain needs that have been satisfied by the host (Dagan *et al.*, 2006). Annotation of the first completed *S.* Typhi genome sequence revealed that $>$ 200 genes have been disrupted or inactivated, representing approximately 5% of its genome (Parkhill *et al.*, 2001), a characteristic that was confirmed by the sequencing of other

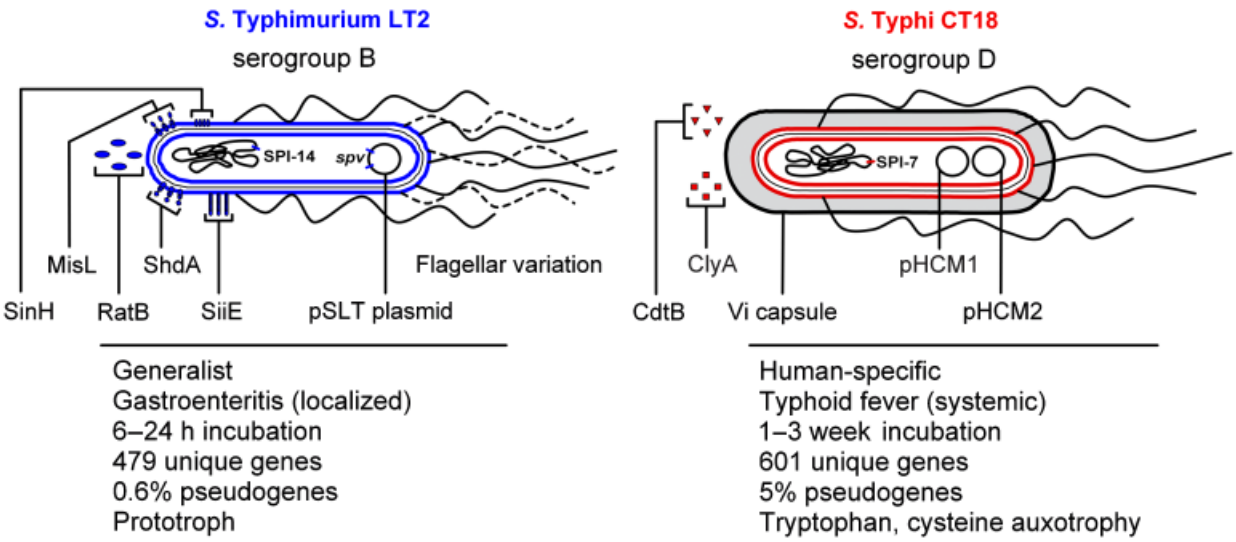

Fig. 1. Major features distinguishing *Salmonella enterica* serovar Typhimurium LT2 from *S.* Typhi CT18.

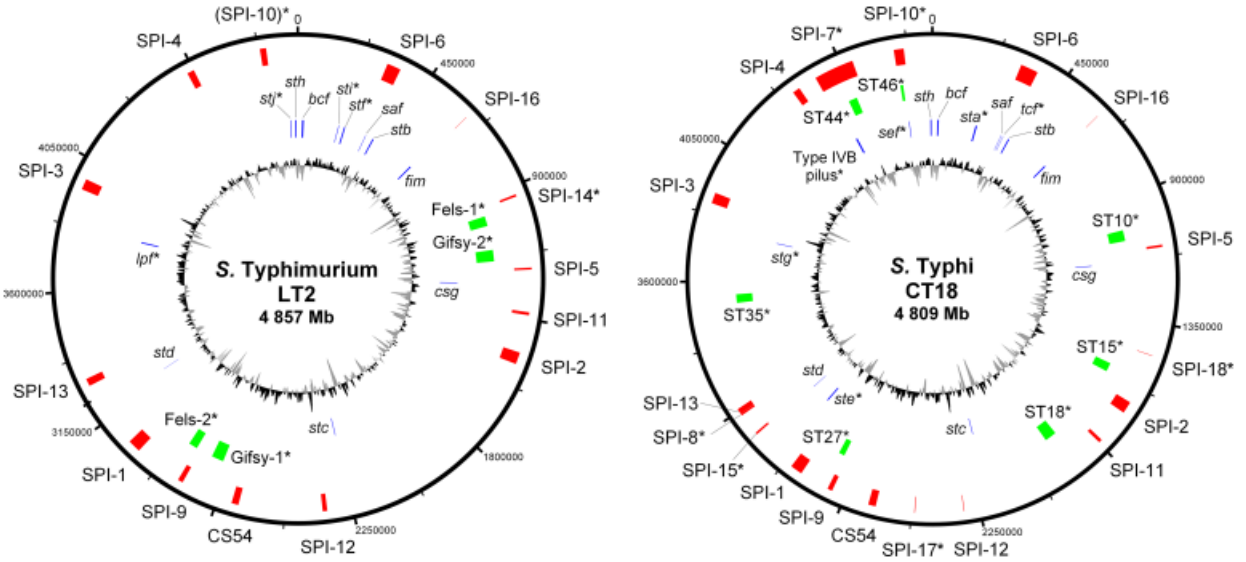

Fig. 2. Circular representation of *Salmonella enterica* serovar Typhimurium LT2 and *S.* Typhi CT18 chromosomes. The circles indicate the localization of the pathogenicity islands (red), the prophages and prophage remnants (green) and fimbrial operons (blue). The inner circle indicates the G+C content (values above the average content are in black, and those below are in grey). Asterisks indicate elements specific to each serovar. The outer scale is marked in bases. The chromosomes were generated using DNAPLOTTER software (Carver *et al.*, 2009).

S. Typhi strains (Deng *et al.*, 2003; Holt *et al.*, 2009). We will point out the different pseudogenes in each of the following sections. Surprisingly, most of the pseudogenes in *S.* Typhi are intact and fully functional in *S.* Typhimurium (McClelland *et al.*, 2001) and could explain in part the loss of host range for serovar *S.* Typhi. Interestingly, many pseudogenes from *S.* Typhi are also conserved in Paratyphi A, a serovar that has the ability to cause enteric fever that afflicts only humans (McClelland *et al.*, 2004; Holt *et al.*, 2009).

Plasmids

Most *S.* Typhimurium strains contain a self-transmissible virulence plasmid (pSLT) of about 90 kb harbouring virulence genes such as the *spv* operon, involved in intramacrophage survival, and the plasmid-encoded fimbriae (*pef*) fimbrial operon (Gulig & Doyle, 1993; Ahmer *et al.*, 1999; Rotger & Casadesús, 1999). When *S.* Typhimurium is cured of the plasmid, virulence in the mouse is decreased (Jones *et al.*, 1982) and can be complemented by the sole addition of the *spv* operon (Gulig *et al.*, 1992) encoding the SpvB toxin (Lesnick *et al.*, 2001). Additionally, *S.* Typhimurium can also carry multidrug-resistance plasmids of high molecular weight (up to 200 kb) and much smaller plasmids (< 20 kb) with unknown functions (Rychlik *et al.*, 2006). The pSLT virulence plasmid is absent in *S.* Typhi strains. In *S.* Typhi, incHI plasmids involved in multiple-drug resistance are commonly found (Maher & Taylor, 1993; Fica *et al.*, 1997; Wain *et al.*, 2003). *Salmonella enterica* serovar Typhi strain CT18 harbours plasmid pHCM1, an incHI1 plasmid of about 218 kb with genes for resistance to antibiotics and heavy metals (Parkhill *et al.*, 2001). *Salmonella enterica* serovar Typhi can also carry cryptic plasmids. *Salmonella enterica* serovar Typhi strain CT18 harbours the cryptic plasmid pHCM2 of about 106 kb whose function is unknown, but it is rarely present in other strains (Parkhill *et al.*, 2001; Kidgell *et al.*, 2002a, b). Additionally, a 27-kb linear plasmid was recently isolated in *S.* Typhi strains originating from Indonesia. This plasmid carries the $fljB^{z66}$ gene, encoding a flagellin antigen known as H:z66 (Baker *et al.*, 2007b). However, no plasmid has been identified yet in *S.* Typhi that has been associated with virulence.

Phages

Integrated bacteriophages represent major loci of genetic diversity in bacterial genomes (Brüssow *et al.*, 2004). *Salmonella* genomes contain several prophages or prophage remnants with similarity to the lambda, Mu, P2 and P4 families (Thomson *et al.*, 2004; Bossi & Figueroa-Bossi, 2005). The contribution of prophages to *S. enterica* virulence has been recognized only recently. Some prophages carry nonessential 'cargo' genes involved in fitness and/or virulence, including several type three secreted effectors (Ehrbar & Hardt, 2005). Each strain of *S.* Typhimurium seems to have a distinct set of prophage elements within its genome. *Salmonella enterica* serovar Typhimurium strain LT2 harbours four prophages, including Gifsy-1, Gifsy-2, Fels-1 and Fels-2 (McClelland *et al.*, 2001; Brüssow *et al.*, 2004). Both the Gifsy-3 and the SopE prophages, found in *S.* Typhimurium strains 14028 and

SL1344, respectively, are absent in *S.* Typhimurium strain LT2 (Figueroa-Bossi *et al.*, 2001; Brüssow *et al.*, 2004; Thomson *et al.*, 2004). *Salmonella enterica* serovar Typhimurium strains SL1344 and 14028 both contain Gifsy-1 and Gifsy-2, but not Fels-1 and Fels-2 (Figueroa-Bossi *et al.*, 2001). *Salmonella enterica* serovar Typhi harbours seven distinct prophage-like elements, spanning > 180 kb, that are generally conserved between strains (Fig. 2) (Thomson *et al.*, 2004). The modular nature of prophage genomes makes a significant contribution to serovar variation and comprises most of the variation in gene content among strains of the same serovar (Boyd *et al.*, 2003; Vernikos *et al.*, 2007).

SPIs

Salmonella has many virulence-associated genes found within clusters in its genome, which are known as SPIs (Mills *et al.*, 1995). Virulence factors encoded by SPI genes tamper with host cellular mechanisms and are thought to dictate the host specificity of the different *S. enterica* serovars (Eswarappa *et al.*, 2008). Many of the SPIs are found next to a tRNA gene (Supporting Information, Fig. S1) and their G+C content differs from the rest of the genome (Fig. 2). Hence, such genomic islands were most likely inserted into the DNA of *Salmonella* by horizontal transfer events, although this explanation remains uncertain (Amavisit *et al.*, 2003). Twenty-one SPIs are known to date in *Salmonella* (McClelland *et al.*, 2001; Parkhill *et al.*, 2001; Chiu *et al.*, 2005; Shah *et al.*, 2005; Vernikos & Parkhill, 2006; Fuentes *et al.*, 2008; Blondel *et al.*, 2009). The *S.* Typhimurium and *S.* Typhi genomes contain 11 common SPIs (SPIs-1 to 6, 9, 11, 12, 13 and 16) (Fig. 2). SPIs-8 and 10 were initially found in *S.* Typhi, and considered as absent in *S.* Typhimurium. However, at both locations in *S.* Typhimurium, there is a completely different set of genes. There is only one SPI specific to *S.* Typhimurium, SPI-14 (Shah *et al.*, 2005), and four SPIs are specific to *S.* Typhi (SPIs-7, 15, 17 and 18) (Fig. 2). SPIs-19, 20 and 21 are absent in both of these serovars and will not be discussed further (Blondel *et al.*, 2009). Even if many of these islands are found in both serovars, differences emerge when comparing equivalent SPIs. In the following section, the genomic differences between *S.* Typhimurium and *S.* Typhi are described for each SPI using *S.* Typhimurium strain LT2 and *S.* Typhi strain CT18 as the genomic references. Amino acid alignments of SPIs between these strains were performed using the XBASE software (Chaudhuri & Pallen, 2006) and can be seen in Fig. S1.

SPI-1 is a 40 kb locus located at centisome 63 encoding a type three secretion system (T3SS) (Mills *et al.*, 1995) and the *sit* metal transport system (Janakiraman & Slauch, 2000). The T3SS is involved in the invasion of nonphagocytic cells and proinflammatory responses (Galán & Curtiss, 1989; Mills *et al.*, 1995; Galán & Collmer, 1999). T3SS are used by the bacteria to inject proteins, called effectors, directly inside the host cells that will act as mediators of cell invasion and modifications contributing to intracellular growth. Effectors can be encoded by genes located inside or outside SPI-1. Genomic comparison confirmed a high degree of identity between the two serovars and revealed the presence of four additional ORFs in *S.* Typhimurium, including the bacterial effector *avrA* (Hardt & Galán, 1997) and three distal ORFs (STM2901, STM2902 and STM2903) encoding putative cytoplasmic proteins (Fig. S1a) (Parkhill *et al.*, 2001). In *S.* Typhi, a partial insertion sequence and transposase are present at the end of the locus. Therefore, the major difference in SPI-1 between both serovars may be at the functional level, as some genes coding effectors located outside SPI-1 are missing (*sspH1*, *steB*) or are pseudogenes (*sopA*, *sopE2* and *slrP*) in *S.* Typhi. All known SPI-1 and SPI-2 effectors of the two serovars are listed in Table S1. Amino acid substitutions in the SipD translocon and the SptP effector were identified between these serovars and may reflect a potential functionality difference (Eswarappa *et al.*, 2008).

SPI-2 is a 40 kb locus inserted next to the *valV* tRNA gene at centisome 30 and encodes a second T3SS, which is involved in intracellular survival (Shea *et al.*, 1996; Hensel *et al.*, 1998). Using comparative genomics, no major differences in SPI-2 were observed between both serovars (Fig. S1b). Three ORFs (STY1735, STY1739 and STY1742) are pseudogenes in *S.* Typhi. These ORFs, however, are not part of the T3SS, but part of a tetrathionate reductase complex. As with SPI-1, some genes encoding effectors in *S.* Typhimurium that are located outside SPI-2 are missing (*sseI*, *sseK1*, *sseK2* and *sseK3*) or are pseudogenes (*sopD2*, *sseJ*) in *S.* Typhi (Table S1). Molecular differences were observed in translocon genes *sseC* and *sseD*, and effectors *sseF* and *sifA* (Eswarappa *et al.*, 2008), reflecting a probable difference in functionality between these serovars.

SPI-3 is a 36 kb locus inserted next to the *selC* tRNA gene located at centisome 82, is involved in intracellular survival and encodes a magnesium transporter (Blanc-Potard & Groisman, 1997). SPI-3 shows extensive variations in its structure in various *S. enterica* serovars and can be divided into three regions (Fig. S1c) (Blanc-Potard *et al.*, 1999; Amavisit *et al.*, 2003). The region found next to the *selC* tRNA gene is where variations between *S.* Typhimurium and *S.* Typhi are the highest, including deletions and insertions. This region contains many pseudogenes in *S.* Typhi: STY4024 (*cigR*), STY4027 (*marT*), STY4030 (*misL*), STY4034, STY4035 and STY4037. A few more pseudogenes in *S.* Typhi are found in the second and third portions of SPI-3, including STY4012, STY4007 and STY4003 (Fig. S1c). In brief, the autotransporter MisL involved in intestinal colonization (Dorsey *et al.*, 2005), its regulator MarT (Tükel *et al.*, 2007) and an unknown putative transcriptional regulator (STY4012) are inactivated in *S.* Typhi.

SPI-4 is a 24 kb fragment located next to a potential tRNA-like gene at centisome 92 (Fig. S1d) and involved in adhesion to epithelial cells (Wong *et al.*, 1998). SPI-4 harbours the *siiABCDEF* gene cluster encoding a type one secretion system (T1SS) for SiiE, a giant nonfimbrial adhesin of 595 kDa (Morgan *et al.*, 2004; Gerlach *et al.*, 2007; Morgan *et al.*, 2007). SiiE mediates a close interaction with microvilli found on the apical side of epithelial cells, thereby aiding efficient translocation of SPI-1 effectors required for apical membrane ruffling (Gerlach *et al.*, 2008). SiiE is encoded by one ORF in *S.* Typhimurium (STM4261), but is segmented into two ORFs in *S.* Typhi (STY4458 and STY4459) because of a stop codon, also present in *S.* Typhi strain Ty2 (Fig. S1d) (Deng *et al.*, 2003). This suggests that *siiE* is a pseudogene in *S.* Typhi (Parkhill *et al.*, 2001; Morgan *et al.*, 2004), which correlates with a loss of function for an adhesin that contributes to intestinal colonization by *S.* Typhimurium (Morgan *et al.*, 2007).

SPI-5 is an island < 8 kb in size, inserted next to the *serT* tRNA gene at centisome 25, and is required for enteropathogenicity (Wood *et al.*, 1998). SPI-5 encodes effectors of both SPI-1 and SPI-2. No difference is observed between the two serovars, except that an additional ORF (STY1114) is predicted to encode a transposase in *S.* Typhi (Fig. S1e).

SPI-6 is located next to the *aspV* tRNA gene at centisome 7 and is a 47 kb island in *S.* Typhimurium (Folkesson *et al.*, 1999; Folkesson *et al.*, 2002), whereas it is rather 59 kb in *S.* Typhi (Parkhill *et al.*, 2001). It was previously shown that the complete deletion of this island reduced the entry of *S.* Typhimurium in Hep2 cells (Folkesson *et al.*, 2002). Located on this island are a type six secretion system (T6SS), the *safABCD* fimbrial gene cluster and the invasin *pagN* (Lambert & Smith, 2008), all present in both serovars (Folkesson *et al.*, 1999; Townsend *et al.*, 2001; Porwollik & McClelland, 2003). A 10 kb fragment downstream of the *saf* operon is found only in *S.* Typhi, and includes probable transposase remnants (STY0343 and STY0344, both pseudogenes), the fimbrial operon *tcfABCD* and genes *tinR* (STY0349) and *tioA* (STY0350) (Fig. S1f) (Folkesson *et al.*, 1999; Townsend *et al.*, 2001; Porwollik & McClelland, 2003). The T6SS of *S.* Typhi contains two pseudogenes, *sciI* (STY0298) and *sciS* (STY0308), and some ORFs are missing or divergent, probably rendering its T6SS nonfunctional. Interestingly, *sciS* was shown to limit the intracellular growth of *S.* Typhimurium in macrophages at a late stage of infection and to decrease virulence in mice (Parsons & Heffron, 2005).

SPI-7 remains the largest island identified to date and is absent in *S.* Typhimurium, but present in *S.* Typhi (Parkhill *et al.*, 2001; Pickard *et al.*, 2003; Bueno *et al.*, 2004). In *S.* Typhi, it is 134 kb in size, corresponding to approximately 150 genes inserted between duplicated *pheU* tRNA sequences (Hansen-Wester & Hensel, 2002; Pickard *et al.*, 2003). This island contains the Vi capsule biosynthesis genes (Hashimoto *et al.*, 1993), whose production is associated with virulence (see section below), a type IVB pilus operon (Zhang *et al.*, 2000) and the SopE prophage (ST44) encoding the SPI-1 effector SopE (Mirold *et al.*, 1999). SopE is also encoded in *S.* Typhimurium's genome, but within the temperate SopE prophage (Hardt *et al.*, 1998) located at a different location (*sopE* is absent in most *S.* Typhimurium strains, including *S.* Typhimurium strain LT2, but present and located on a prophage in *S.* Typhimurium strains SL1344 and 14028) (Hardt *et al.*, 1998; Mirold *et al.*, 1999; Pelludat *et al.*, 2003). At the SPI-7 location in *S.* Typhimurium LT2, we find a single complete *pheU* tRNA sequence and STM4320 (a putative *merR* family bacterial regulatory protein) (Fig. S1g).

SPI-8 is an 8 kb DNA fragment found next to the *pheV* tRNA gene that is part of SPI-13 and will be discussed in that section (Fig. S1l) (Parkhill *et al.*, 2001; Hensel, 2004).

SPI-9 is a 16 kb locus present in both serovars (Fig. S1h). This island contains three genes encoding for a T1SS and one for a large protein, sharing an overall 40% nucleotide identity to *siiCDEF* genes from SPI-4 (Morgan *et al.*, 2004, 2007). The large protein-coding ORF (STM2689) in *S.* Typhimurium strain LT2 was first suggested to be a pseudogene (McClelland *et al.*, 2001; Morgan *et al.*, 2004). However, a subsequent study showed an undisrupted gene coding a putative 386 kDa product renamed BapA (Latasa *et al.*, 2005).

SPI-10 is an island found next to the *leuX* tRNA gene at centisome 93. This locus is completely different in each serovar and has been termed SPI-10 only in *S.* Typhi. In *S.* Typhimurium, it is substituted by a 20 kb uncharacterized island without any SPI annotation (Fig. S1i), comprising functionally unrelated genes that share little homology to sequences from the genomic databases (Edwards *et al.*, 2001; Bishop *et al.*, 2005). However, a possible relationship of these genes with DNA repair has been proposed (Porwollik & McClelland, 2003). Deletion of this island in *S.* Typhimurium strain 14028 caused attenuation of virulence in mice (Haneda *et al.*, 2009). In *S.* Typhi's genome, this island corresponds to a 33 kb fragment (Parkhill *et al.*, 2001) carrying a full P4-related prophage, termed ST46 (Edwards *et al.*, 2001; Thomson *et al.*, 2004; Bishop *et al.*, 2005). ST46 harbours the *prpZ* cluster as cargo genes encoding eukaryotic-type Ser/Thr protein kinases and phosphatases involved in *S.* Typhi survival in macrophages (Faucher *et al.*, 2008). There is also a complete, but inactivated *sefABCDR* (*S.* Enteritidis fimbriae) fimbrial operon (Fig S1i). Many pseudogenes are found in *S.* Typhi: STY4835 (IS*1230*), STY4836 (*sefA*), STY4839 (*sefD*), STY4841 (*sefR*), STY4845 (a thiol : disulphide interchange protein) and STY4848 (putative transposase) (Fig. S1i). Interestingly, ORFs STY4842–4846 of *S.* Typhi are homologues to *S.* Typhimurium genes located on the virulence plasmid, including *srgA* (Rodríguez-Peña *et al.*, 1997). *srgA* encodes a functional disulphide oxidoreductase in *S.* Typhimurium and is a

pseudogene in *S.* Typhi (STY4845) (Bouwman *et al.*, 2003). It was shown that SrgA acts in concert with DsbA, another disulphide oxidoreductase, to target SipA (a SPI-2 effector), and that an *srgA dsbA* double mutant had a stronger attenuation than either single mutants, with a level of attenuation similar to a SPI-2 mutant (Miki *et al.*, 2004).

SPI-11 was initially identified in the genome sequencing of serovar Choleraesuis as a 14 kb fragment inserted next to the Gifsy-1 prophage (Chiu *et al.*, 2005). This SPI is shorter in *S.* Typhimurium (6.7 kb) and in *S.* Typhi (10 kb) (Fig. S1j). SPI-11 includes the *phoP*-activated genes *pagD* and *pagC* involved in intramacrophage survival (Miller *et al.*, 1989; Gunn *et al.*, 1995). The putative envelope lipoprotein *envF* is absent in *S.* Typhi, while six additional ORFs (STY1884–1891), including the typhoid toxin *cdtB*, are present in *S.* Typhi (Fig. S1j) (Spanò *et al.*, 2008).

SPI-12, located next to the *proL* tRNA gene at centisome 48, is 15.8 kb long in *S.* Typhimurium and 6.3 kb long in *S.* Typhi (Fig. S1k) (Hansen-Wester & Hensel, 2002). It contains the effector SspH2 (Miao *et al.*, 1999). The additional 9.5 kb fragment in *S.* Typhimurium contains 11 ORFs, which include some putative and phage-associated genes as well as *oafA*, encoding a *Salmonella*-specific gene for O-antigen acetylase (Fig. S1k) (Slauch *et al.*, 1996; Hansen-Wester & Hensel, 2002). SPI-12 was shown to be required for systemic infection of mice in *S.* Typhimurium strain 14028 (Haneda *et al.*, 2009). In *S.* Typhi, three ORFs are pseudogenes (STY2466a, STY2468 and STY2469), leaving only the *sspH2* gene as functional on this island.

SPI-13 was initially identified in serovar Gallinarum (Shah *et al.*, 2005). This 25 kb gene cluster is found next to the *pheV* tRNA gene at centisome 67 in *S.* Typhimurium and in *S.* Typhi. However, an 8 kb portion is different in each serovar and corresponds to SPI-8 only in *S.* Typhi (Fig. S1l). In *S.* Typhimurium, this region contains the ORFs STM3117 to STM3123, a cluster unique to *S.* Typhimurium, coding genes for a putative lyase, hydrolase, oxidase, arylsulphatase and arylsulphatase regulator as well as two putative LysR family transcriptional regulators (Fig. S1l). In strain *S.* Typhimurium 14028, STM3117–STM3121 are novel virulence-associated genes, as they were shown to be involved in systemic infection of mice (Haneda *et al.*, 2009) and replication inside murine macrophages (Shi *et al.*, 2006). In *S.* Typhi, the virulence function of SPI-8 is unknown and it harbours two bacteriocin immunity proteins (STY3281 and STY3283) and four pseudogenes (Fig. S1l) (Parkhill *et al.*, 2001). The 17 kb conserved portion of SPI-13 has not been shown to contribute to virulence (Haneda *et al.*, 2009).

SPI-14 corresponds to 9 kb present in *S.* Typhimurium at centisome 19 and is absent in *S.* Typhi (Shah *et al.*, 2005; Morgan, 2007). It harbours seven ORFs encoding putative cytoplasmic proteins (Fig. S1m). The function of genes on this island is unknown, but gene upregulation was observed in macrophages infected by *S.* Typhimurium strain SL1344 (Eriksson *et al.*, 2003).

SPI-15 is a 6.5 kb island of five ORFs encoding hypothetical proteins, is inserted near the *glyU* tRNA gene in *S.* Typhi and is absent in *S.* Typhimurium (Fig. S1n) (Vernikos & Parkhill, 2006). Different genes are found at the same location in *S.* Typhi strain Ty2 (Fig. S1n) (Vernikos & Parkhill, 2006). SPI-15, as well as SPI-16 and 17, were identified by bioinformatic work (Vernikos & Parkhill, 2006).

SPI-16 is found in *S.* Typhimurium and *S.* Typhi as a 4.5 kb fragment inserted next to an *argU* tRNA site, and encodes five or seven ORFs, respectively, four of which are pseudogenes in *S.* Typhi (Fig. S1o). The three remaining ORFs show a high level of identity with P22 phage genes involved in seroconversion (Vernikos & Parkhill, 2006) and were suggested to mediate O-antigen glycosylation (Mavris *et al.*, 1997; Guan *et al.*, 1999) and cell surface variation (Allison & Verma, 2000; Bogomolnaya *et al.*, 2008). These ORFs (genes *yfdH*, *rfbI* and STM0557) were required for the intestinal persistence of *S.* Typhimurium in mice (Bogomolnaya *et al.*, 2008).

SPI-17 is a 5 kb island encoding six ORFs inserted next to an *argW* tRNA site and is absent in *S.* Typhimurium, but present in *S.* Typhi (Fig. S1p) (Vernikos & Parkhill, 2006). Seroconversion genes homologous to P22 phage are present and showed high homology to genes of SPI-16, including a putative lipopolysaccharide modification acyltransferase. Most of these genes (four) are pseudogenes in *S.* Typhi (Fig. S1p).

SPI-18 was recently identified in *S.* Typhi as a 2.3 kb fragment harbouring only two ORFs: STY1498 and STY1499 (Fig. S1q) (Fuentes *et al.*, 2008). *clyA* (STY1498), also known as *hlyE* or *sheA*, encodes a 34 kDa pore-forming secreted cytolysin found in *E. coli* and *S. enterica* serovars Typhi and Paratyphi A (del Castillo *et al.*, 1997; Green & Baldwin, 1997; Oscarsson *et al.*, 1999, 2002). *clyA* is important for invasion of human epithelial cells *in vitro*, with its heterologous expression in *S.* Typhimurium leading to colonization of deep organs in a murine model (Fuentes *et al.*, 2008). *taiA* (STY1499) is a secreted 27 kDa invasin that increases bacterial uptake by human macrophages (Faucher *et al.*, 2009). Both genes are part of a common operon and are controlled by the virulence-related regulator PhoP (Faucher *et al.*, 2009).

Other pathogenicity islands are found in the *S.* Typhimurium and *S.* Typhi genomes and have not been identified as SPIs, but encode genes responsible for virulence in the host, such as CS54. The CS54 island is a 25 kb region found between *xseA* and *yfgJ* at centisome 54 in *S.* Typhimurium (Kingsley *et al.*, 2003) and *S.* Typhi (Fig. S1r). Five genes are found within this island, which are *shdA*, *ratB*, *ratA*, *sinI* and *sinH* (*sivH*). In *S.* Typhimurium, ShdA was shown to be an outer membrane protein of the autotransporter family that binds fibronectin, RatB is a predicted secreted protein of unknown function and SinH is a putative outer membrane

protein (Kingsley & Bäumler, 2002; Kingsley *et al.*, 2003; Abd El Ghany *et al.*, 2007). *shdA*, *ratB* and *sinH* (*sivH*) are all implicated in intestinal colonization of BALB/c mice by *S.* Typhimurium, but are all pseudogenes in *S.* Typhi (Kingsley *et al.*, 2003).

Other virulence factors

Fimbriae

Fimbriae or pili are proteinous structures found on bacteria that can mediate interaction with cells. Fimbriae are normally specific to a receptor and can be used at different critical times during the infection. Each serovar harbours a unique combination of fimbrial operons (Fig. 2). Whole-genome sequence analysis revealed eight putative fimbrial operons shared by both *S.* Typhimurium and *S.* Typhi [*bcf*, *csg* (*agf*), *fim*, *saf*, *stb*, *stc*, *std*, *sth*] (McClelland *et al.*, 2001; Parkhill *et al.*, 2001). *Salmonella enterica* serovar Typhimurium possesses five unique fimbrial sequences known as *lpf*, *stf*, *pef*, *sti* and *stj* (McClelland *et al.*, 2001). These unique fimbriae were not involved in systemic colonization of the spleen, and Lpf was shown to be involved in intestinal colonization of mice (Weening *et al.*, 2005). A type IVB pilus located on SPI-7 is only found within the *S.* Typhi genome, along with five other unique fimbrial operons (*sef*, *sta*, *ste*, *stg* and *tcf*) (Parkhill *et al.*, 2001). For the majority of these fimbriae, little is known about their true function, expression conditions or their importance for virulence during infection. Type IV pili are used by Typhi for adhesion to human monocytes and epithelial cells by interaction with the cystic fibrosis transmembrane conductance regulator receptor (Pier *et al.*, 1998; Zhang *et al.*, 2000; Tsui *et al.*, 2003; Pan *et al.*, 2005). Tcf was recognized by human sera from patients with typhoid (Harris *et al.*, 2006) and Stg mediates adherence to epithelial cells and reduces phagocytosis (Forest *et al.*, 2007). All fimbrial operons are intact in *S.* Typhimurium strain LT2, although pseudogenes are found within six fimbrial operons of *S.* Typhi strain CT18 (*fimI*, *safE*, *sefA*, *sefD*, *bcfC*, *steA*, *stgC*, *sthC*, *sthE*) (Townsend *et al.*, 2001) (http://www.pseudogene.org/cgi-bin/db-gen.cgi?type=Prokaryote). The unique repertoire of fimbrial adhesin systems may explain in part some differences observed between the host tropism colonization niches of these two serovars.

Flagella

In *Salmonella*, the major subunit of flagella is usually encoded by *fliC* or *fljB*, which correspond to the H1 and H2 variants of the H antigen, respectively (Silverman & Simon, 1980). Only one type of flagellin can be expressed at a specific time by a mechanism known as phase variation (Lederberg & Iino, 1956; Simon *et al.*, 1980). This antigenic variation can be observed in *S.* Typhimurium, but most *S.* Typhi strains are considered monophasic, as they lack a corresponding *fljB* locus (Frankel *et al.*, 1989). However, some *S.* Typhi isolates from Indonesia contain a linear plasmid encoding a novel flagellin, $fljB^{z66}$, but reversion to *fliC* is considered irreversible due to a deletion (Baker *et al.*, 2007a). *fliB*, involved in methylation of the flagellin in *S.* Typhimurium, is a pseudogene in *S.* Typhi (Parkhill *et al.*, 2001).

Vi antigen

The Vi antigen is a polysaccharidic capsule absent in *S.* Typhimurium and present in *S.* Typhi. Vi is important for virulence and is controlled by two loci: *viaA* and *viaB* (Kolyva *et al.*, 1992). The *viaB* locus located on SPI-7 is composed of two operons: *tviABCDE* and *vexABCDE*. The Vi capsule causes several differences between *S.* Typhimurium and *S.* Typhi at the level of the host's response to infection. The Vi capsule is associated with inhibition of complement activation, resistance to serum and to phagocytosis and is involved in survival inside phagocytes (Looney & Steigbigel, 1986; Hirose *et al.*, 1997; Miyake *et al.*, 1998). The *viaB* locus lowers the invasiveness of the bacteria towards epithelial cells, as *viaB* mutants are superinvasive (Arricau *et al.*, 1998; Zhao *et al.*, 2001), and *S.* Typhimurium harbouring the *viaB* locus is less invasive (Haneda *et al.*, 2009). TviA avoids interleukin-8 production in the intestinal mucosa by repressing flagellin secretion, which reduces the recognition and activation of Toll-like receptor (TLR)-5 (Raffatellu *et al.*, 2005; Winter *et al.*, 2008). Vi also prevents the recognition of lipopolysaccharide by TLR-4 and reduces inflammation in the intestinal mucosa (Sharma & Qadri, 2004; Wilson *et al.*, 2008). *Salmonella enterica* serovar Typhimurium sets off an immune response, which causes inflammation characterized by an important neutrophil influx that may be the result of its lack of capsule. Thus, Vi allows *S.* Typhi to disseminate systemically in its human host by crossing intestinal cells without activating the immune response, promotes resistance to killing by serum and contributes to survival inside phagocytes (Raffatellu *et al.*, 2006). Vi is a protective antigen and the actual constituent of the parenteral typhoid fever vaccine.

Conclusions

Acquisition and loss of genetic material play an important role in bacterial evolution. Here, we have described the major genetic differences between *S.* Typhimurium and *S.* Typhi, two important *S. enterica* serovars associated with distinct diseases in humans (Fig. 1). Gene degradation in *S.* Typhi may be responsible for its human host restriction, but factors contributing to its systemic dispersion and survival during typhoid may be multiple and scattered, which

complicates the identification of genomic regions that reflect differences in habitat and lifestyle.

Each serovar is distinguished by its own repertoire of virulence factors, whether surface expressed or secreted, that leads to specific diseases or hosts. The bacterial cell surface is different between the two serovars, as represented by various O:H:K antigens. Lipopolysaccharide differences (O antigen) allowed the classification of *S.* Typhimurium in serogroup B, while *S.* Typhi belongs to serogroup D. Only *S.* Typhimurium is capable of phase variation of its H antigen, by differential expression of two flagella subunits. The most important feature is undoubtedly the presence of a polysaccharidic capsule (K antigen) specific to *S.* Typhi, the Vi antigen. However, it is also interesting to note that some *S.* Typhi strains and *S.* Paratyphi A lack the Vi antigen, but both cause a disease very similar to *S.* Typhi in the human host (McClelland *et al.*, 2004; Baker *et al.*, 2005).

Virulence factors can be secreted using the general secretion machinery of the bacteria or by specific systems, such as the T3SS used to inject proteins directly into the host. No major differences were observed in both T3SS (Fig. S1a,b), but some of the effectors were missing in *S.* Typhi (Table S1). However, the T6SS included on SPI-6 is probably inactivated in *S.* Typhi by the presence of pseudogenes. Some toxins were specific to *S.* Typhimurium, such as SpvB present on the virulence plasmid. On the other hand, the CdtB and ClyA toxins are only produced by *S.* Typhi.

Interestingly, most of the genes involved in intestinal colonization identified in *S.* Typhimurium are inactivated in *S.* Typhi. These genes encode autotransporters MisL and ShdA, adhesin SiiE, secreted protein RatB, putative outer membrane protein SinH and Lpf fimbriae (Fig. 1), suggesting that they are not needed by *S.* Typhi in the human host. This particular divergence is further acknowledged when looking at some work involving vaccine development (DiPetrillo *et al.*, 1999; Angelakopoulos & Hohmann, 2000; Hindle *et al.*, 2002). *Salmonella enterica* serovar Typhimurium and *S.* Typhi live-attenuated vaccine strains, both modified with the same genetic deletions, did not show the same level of intestinal colonization when administered orally to human volunteers. Prolonged bacterial shedding by the host was observed over time with *S.* Typhimurium, but not with *S.* Typhi. Thus, precautions must be taken when extrapolating the *S.* Typhimurium data to *S.* Typhi. Many clinical trials investigating novel *S.* Typhi vaccine strains harbouring mutations that render *S.* Typhimurium avirulent and immunogenic in mice led to disappointing results at the attenuation level when administered to humans (Hone *et al.*, 1988; Tacket *et al.*, 1992a, b).

The completion of additional genome sequencing projects of other *Salmonella* serovars or strains will contribute considerably to our understanding of niche adaptation and bacterial evolution in general, as well as conceiving the molecular basis of epidemics and how new virulent strains emerge. However, the availability of whole-genome sequences of several strains of different *S. enterica* serovars has not revealed any explanation that correlates with their specific niches and fitness, which suggests that the answer is probably already under our noses . . ..

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Genomic comparison of pathogenicity islands from *Salmonella enterica* serovar Typhimurium LT2 and *Salmonella enterica* serovar Typhi CT18.
Table S1. List of SPI-1 and SPI-2 effectors from *Salmonella enterica* serovar Typhimurium LT2 and *Salmonella enterica* serovar Typhi CT18.

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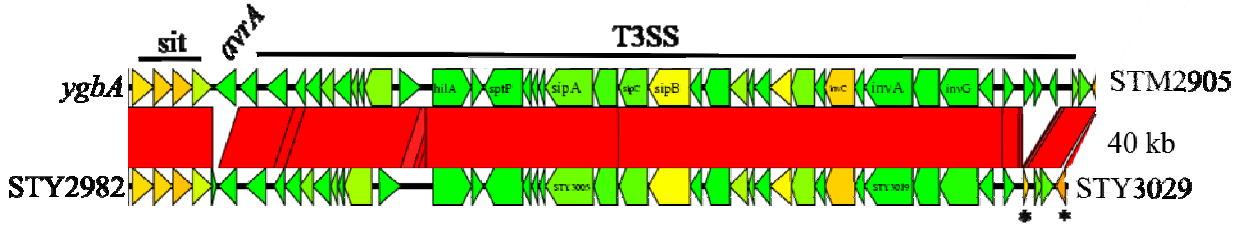
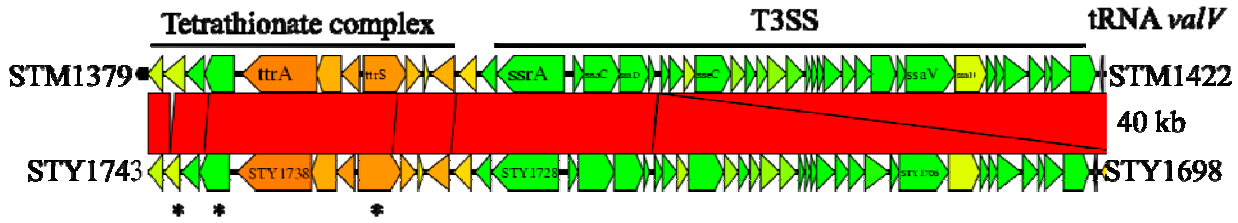
Figures supplémentaires de l'article S2. Sabbagh, S. C., **Forest, C. G.**, Lepage, C., Leclerc, J. M. and F. Daigle. 2010. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. FEMS Microbiol. Lett., **305:**1-13.

Fig. S1. Genomic comparison of pathogenicity islands from *S.* Typhimurium LT2 and *S.* Typhi CT18. Pseudogenes in *S.* Typhi are represented by an asterisk (*). Amino acid sequence alignments of pathogenicity islands were generated using xBASE (promer) (30).

a) SPI-1



b) SPI-2



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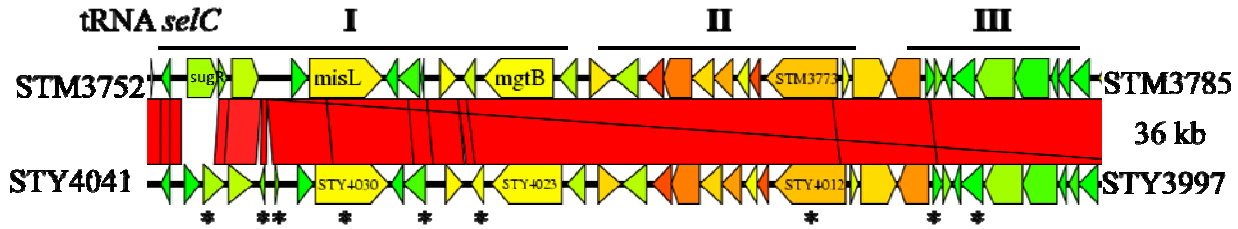
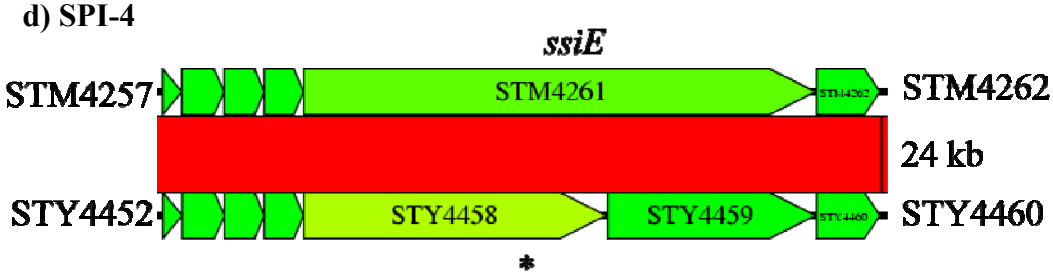
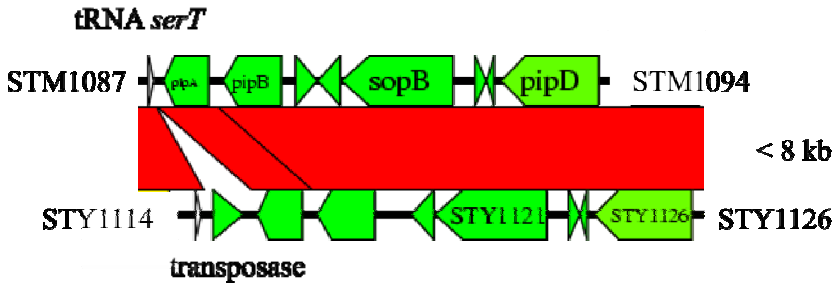


d) SPI-4



X

e) SPI-5



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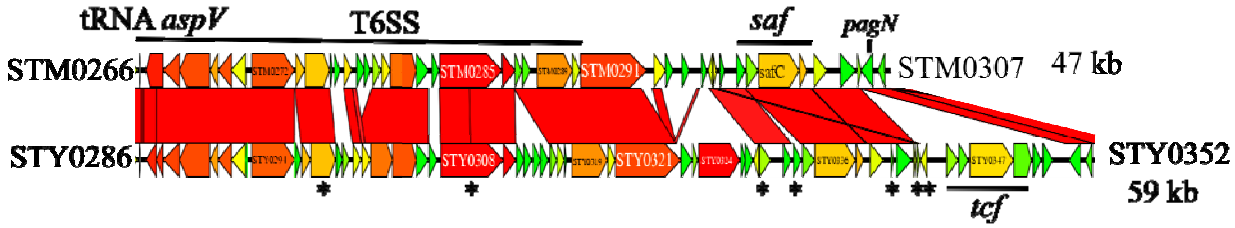

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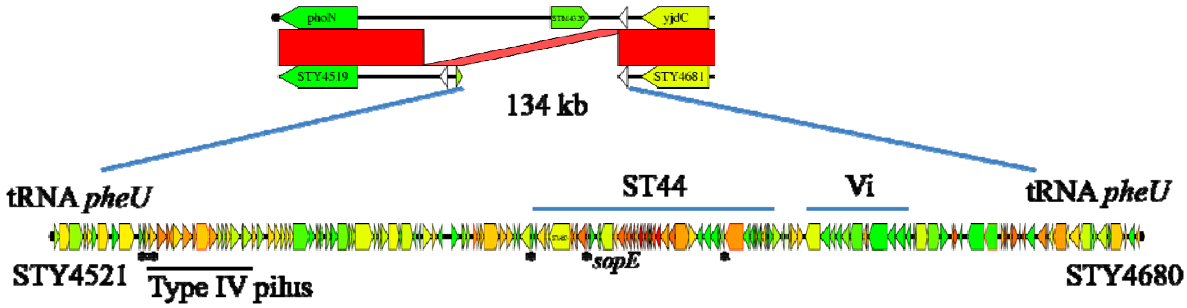

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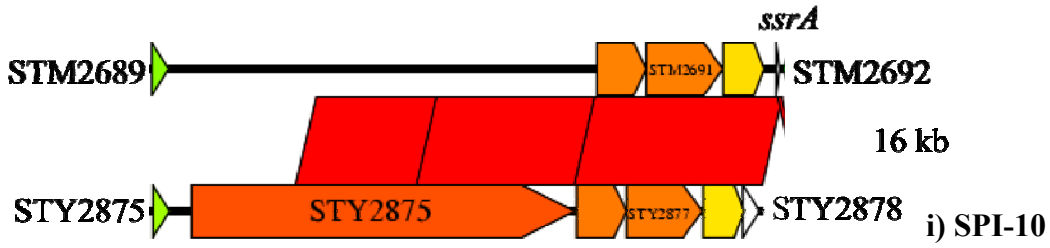

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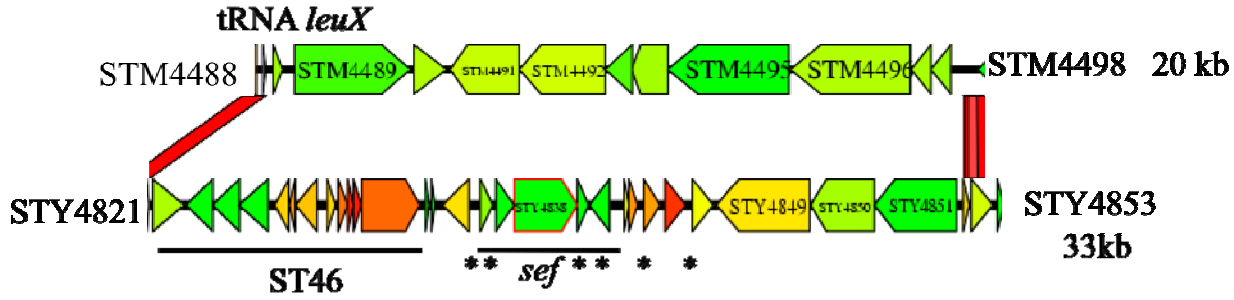

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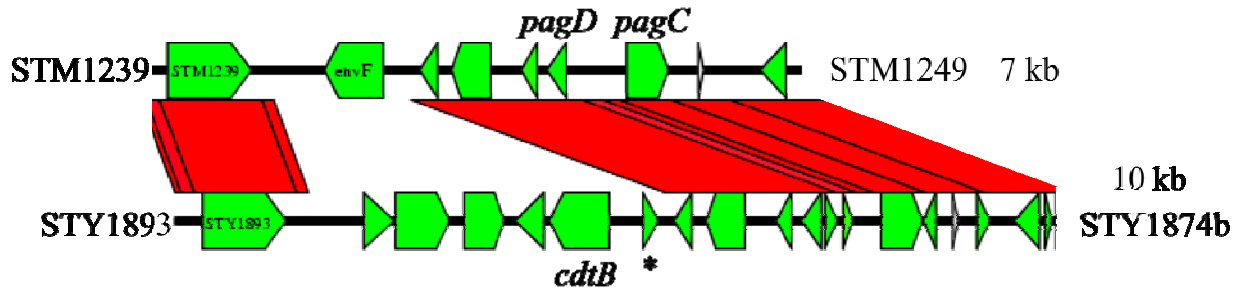

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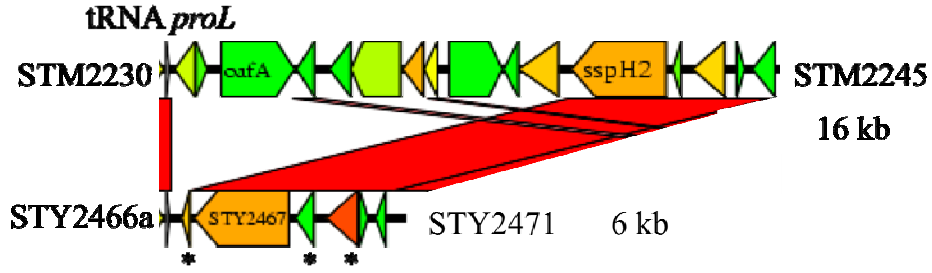
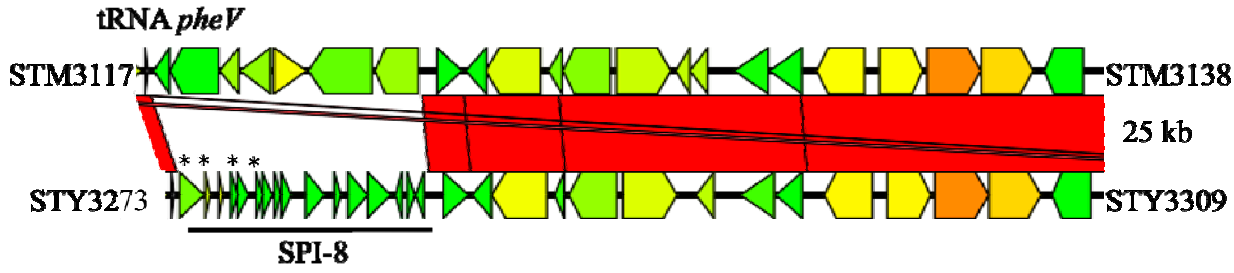
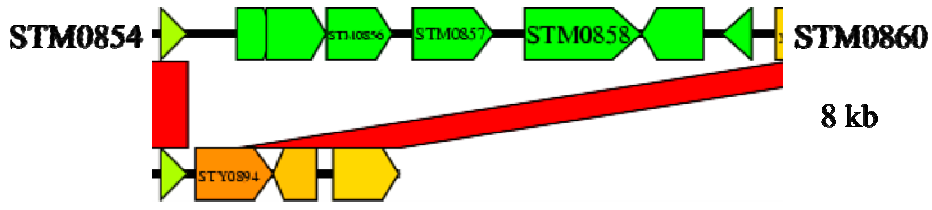


l) SPI-13

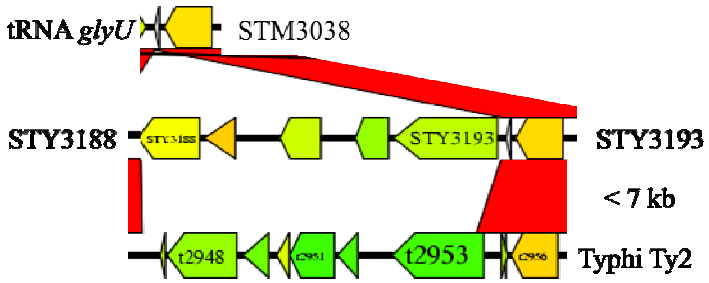


m) SPI-14



XII

n) SPI-15



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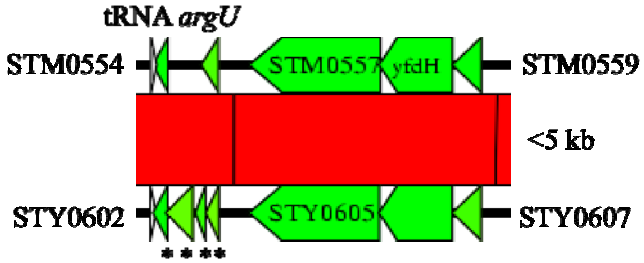
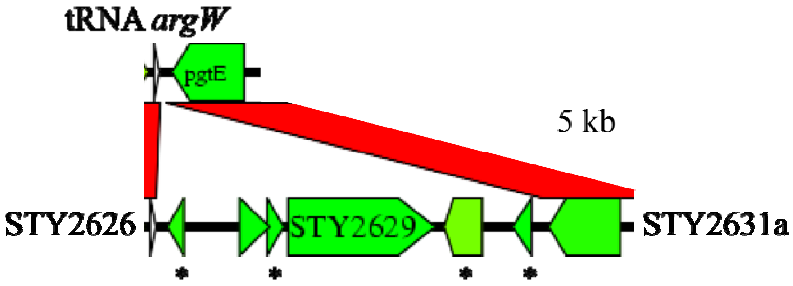
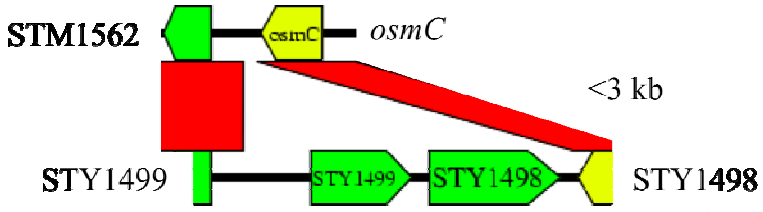


p) SPI-17



q) SPI-18



r) CS54

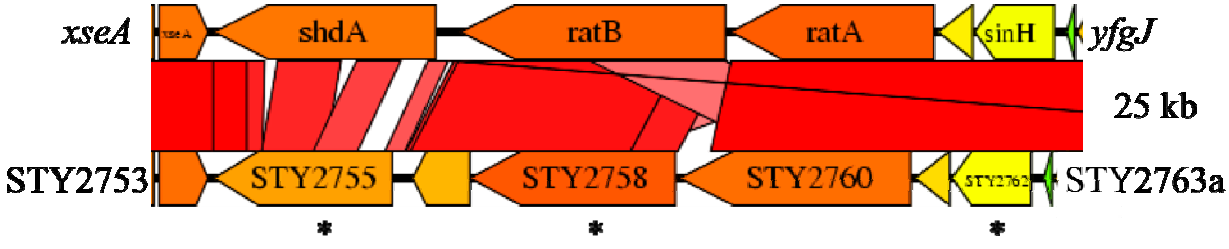


Table S1. List of SPI-1 and SPI-2 effectors from *S.* Typhimurium LT2 and *S.* Typhi CT18.

Effectors	LT2	CT18	% identity	% homology
SPI1 T3SS				
AvrA	STM2865	A[e]	-[a]	-
SipA (SspA)	STM2882	STY3005	97	98
SipB (SspB)	STM2885	STY3008	99	99
SipC (SspC)	STM2884	STY3007	99	99
SipD (SspD)	STM2883	STY3006	87	93
SopA	STM2066	(STY2275)[b]	-	-
SopB (SigD)	STM1091	STY1121	98	98
SopE	A[c]	STY4609	-	-
SopE2	STM1855	(STY1987)	-	-
SptP	STM2878	STY3001	94	96
SopD	STM2945	STY3073	98	99
SteA	STM1583	STY1482	88	93
SteB	STM1629	A	-	-
SPI2 T3SS				
GogB	STM2584	A	-	-
PipB	STM1088	STY1117	97	98
PipB2	STM2780	STY2897	91	96
SifA	STM1224	STY1264	93	95
SifB	STM1602	STY1462	98	99
SopD2	STM0972	(STY0971)	-	-
SpiC (SsaB)	STM1393	STY1727	100	100
SseF	STM1404	STY1716	96	96
SseG	STM1405	STY1715	98	99
SseI	STM1051	A	-	-
SseJ	STM1631	(STY1439a)	-	-
SseK1	STM4157	A	-	-
SseK2	STM2137	A	-	-
SseK3	A[d]	A	-	-
SseL	STM2287	STY2517	93	97
SspH2	STM2241	STY2467	99	99
SteC	STM1698	STY1353	91	95
SpvB	pSLT039	A	-	-
SpvC	pSLT038	A	-	-
Translocated by both SPI1 and SPI2 T3SS				
SlrP	STM0800	(STY0833)	-	-
SspH1	A	A	-	-

[a]No BlastP can be done. [b]ORFs in parentheses and gray shade are pseudogenes.
[c]Only present in ATCC strain 14028 on Gifsy-3 prophage.
[d]Present on ST64B prophage. [e]A, absent from chromosome.

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A novel PhoP-regulated locus encoding the cytolysin ClyA and the secreted invasin TaiA of *Salmonella enterica* serovar Typhi is involved in virulence

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Salmonella enterica serovar Typhi causes a human-restricted systemic infection called typhoid fever. We have identified a Typhi genomic region encoding two ORFs, STY1498 and STY1499, that are expressed during infection of human macrophages and organized in an operon. STY1498 corresponds to *clyA*, which encodes a pore-forming cytolysin, and STY1499 encodes a 27 kDa protein, without any attributed function, which we have named TaiA (Typhi-associated invasin A). In order to evaluate the roles of these genes in Typhi pathogenesis, isogenic Typhi strains harbouring a non-polar mutation of either *clyA* or *taiA* were constructed. In macrophages, *taiA* was involved in increasing phagocytosis, as *taiA* deletion reduced bacterial uptake, whereas *clyA* reduced or controlled bacterial growth, as *clyA* deletion enhanced Typhi survival within macrophages without affecting cytotoxicity. In epithelial cells, deletion of *taiA* had no effect on invasion, whereas deletion of *clyA* enhanced the Typhi invasion rate, and reduced cytotoxicity. Overexpression of *taiA* in Typhi or in *Escherichia coli* resulted in a higher invasion rate of epithelial cells. We have demonstrated that TaiA is secreted independently of both the *Salmonella* pathogenicity island (SPI)-1 and the SPI-2 type three secretion systems. We have shown that this operon is regulated by the virulence-associated regulator PhoP. Moreover, our results revealed that products of this operon might be involved in promoting the use of macrophages as a sheltered reservoir for Typhi and allowing long-term persistence inside the host.

INTRODUCTION

The genus *Salmonella* is composed of two distinct species, *Salmonella bongori* and *Salmonella enterica*. While *S. bongori* is rarely involved in human infections, *S. enterica* is a major human pathogen. Out of the 2000 serovars of *S. enterica*, only a small fraction is associated with human infections (Porwollik *et al.*, 2004). Serovars Typhimurium and Enteritidis cause a localized infection, gastroenteritis, whereas serovar Typhi causes a severe systemic infection called typhoid fever, which kills an estimated 600 000 people annually (Parry *et al.*, 2002). Because Typhi is restricted to humans, Typhimurium has been used for many years to study typhoid fever pathogenesis using a murine infection model in which Typhimurium causes a systemic infection. This model has been crucial in understanding systemic infections by *Salmonella*.

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Abbreviations: DAP, diaminopimelic acid; HA, haemagglutinin; LDH, lactate dehydrogenase; OMV, outer membrane vesicle; qPCR, quantitative PCR; SCV, *Salmonella*-containing vacuole; SPI, *Salmonella* pathogenicity island; T3SS, type three secretion system.

S. enterica and *S. bongori* possess a type three secretion system (T3SS), encoded by *Salmonella* pathogenicity island 1 (SPI-1), which mediates invasion of host cells (Galan, 1999; Marcus *et al.*, 2000). Only *S. enterica* possesses a second T3SS located in SPI-2, which is required for survival inside macrophages and the infection of mammalian hosts (Ochman & Groisman, 1996; Ochman *et al.*, 1996). The T3SS injects bacterial proteins directly into the host cell and disturbs normal cell function. Induction of the SPI-1-encoded genes requires high osmolarity and low aeration, conditions present in the small intestine where the SPI-1 T3SS initiates cell invasion (Altier, 2005; Lostroh & Lee, 2001). SPI-2 T3SS genes are induced by low concentrations of magnesium and phosphate and an acidic pH (Beuzon *et al.*, 1999; Coombes *et al.*, 2004; Deiwick *et al.*, 1999). SPI-2-translocated effectors are involved in modification of the *Salmonella*-containing vacuole (SCV) and inhibition of lysosome fusion (Kuhle & Hensel, 2004). In *Salmonella*, regulation of a multitude of genes involved in intracellular survival, phagosome alteration, invasion, lipid A modification, and resistance to antimicrobial peptides, including the SPI-2 T3SS, is mediated by the PhoPQ two-component system (Prost & Miller, 2008).

Infections with Typhi are characterized by a long incubation period (7–14 days), a three-week period of symptoms, including fever and malaise, and mild intestinal inflammation (Connor & Schwartz, 2005). In contrast, Typhimurium infections in humans have a shorter incubation period (10–72 h), a shorter symptomatic period (<10 days) and produce strong intestinal inflammation (Santos *et al.*, 2001). Therefore, Typhi is likely to possess and use unique virulence factors to systemically infect humans. It has been shown that Typhi is able to survive better inside human macrophages than Typhimurium and that Typhimurium survives better inside murine macrophages than Typhi (Schwan *et al.*, 2000). The highest level of survival in macrophages seems to correlate with the host in which each serovar is able to cause a systemic infection. Survival in host macrophages is known to have a great effect on virulence (Fields *et al.*, 1986), and host macrophages have been shown to be the reservoir of *Salmonella* during systemic disease (Richter-Dahlfors *et al.*, 1997). Therefore, in the absence of an adequate animal model to study typhoid fever, it is important to focus our studies on Typhi interaction with host cells in order to better characterize its mechanisms of pathogenicity.

Transcriptomic studies of Typhi have identified a group of 117 genes that are induced continuously within infected human macrophages (Faucher *et al.*, 2006). This group includes many genes of the SPI-2 T3SS, several genes involved in antimicrobial peptide resistance and many genes with no associated function (Faucher *et al.*, 2006). Some of these genes are absent from the Typhimurium genome, including *clyA* (STY1498) and STY1499, which are two contiguous genes. Recently, these two Typhi proteins were detected under conditions that induce SPI-2, and which are thought to mimic the intracellular environment of macrophages (Ansong *et al.*, 2008).

ClyA (HlyE/SheA) is a well-characterized pore-forming cytolysin found in serovars Typhi and Paratyphi A, and in some *Escherichia coli* strains (Oscarsson *et al.*, 2002). ClyA monomers are exported in outer membrane vesicles (OMVs). *E. coli* cells expressing *clyA* are cytotoxic for mammalian cells (Lai *et al.*, 2000; Oscarsson *et al.*, 1999). A specific antibody response toward ClyA during human infection by Typhi or Paratyphi was recently reported, which indicates that ClyA is expressed *in vivo* (von Rhein *et al.*, 2006). However, direct evidence of ClyA playing a role in Typhi pathogenesis has not been reported yet. STY1499 is a putative ORF of unknown function, which we have named *taiA* (Typhi associated invasin A). Expression of this gene cluster inside human macrophages suggests an involvement in Typhi pathogenesis. To investigate this possibility, non-polar mutant strains of *clyA* and *taiA* were constructed and their interaction with human cells was characterized.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in Luria–Bertani (LB) broth. For the invasion assay or SPI-1 induction, bacteria were grown overnight in LB containing 0.3 M NaCl (LB NaCl) without aeration. For SPI-2 induction, low-phosphate, low-magnesium medium (LPM), pH 5.8, was used, as described by Coombes *et al.* (2004). N medium, pH 5.8, with 10 μM $MgCl_2$ was used for PhoP activation, and N medium, pH 7.4, with 10 mM $MgCl_2$ was used for PhoP inactivation (Snavely *et al.*, 1991). When required, antibiotics, amino acids or supplements were added at the following concentrations: 50 μg ampicilin ml^{-1}, 50 μg diaminopimelic acid (DAP) ml^{-1}, 34 μg chloramphenicol ml^{-1}, 22 μg tryptophan ml^{-1}, 22 μg cysteine ml^{-1} and 22 μg arginine ml^{-1}. Transformation of bacterial strains was routinely done by using the calcium/manganese-based (CCMB) or electroporation methods, as described by O'Callaghan & Charbit (1990).

Generation of mutants and complementation. To generate non-polar mutations of *taiA* and *clyA*, the overlap-extension PCR method described by Basso *et al.* (2002) was used. For *taiA*, fragments were amplified with primers STY1499-BF and STY1499-BR (Table 2) for the 5′ end of the gene, and primers STY1499-EF and STY1499-ER for the 3′ end of the gene. These two fragments were ligated in a second PCR by using the external primers STY1499-BF and STY1499-ER. The resulting fragment, containing a 318 bp internal deletion, was digested with *Bam*HI and *Xba*I and ligated into suicide vector pMEG375 (Kaniga *et al.*, 1998). The resulting plasmid pSIF024 was conjugated from *E. coli* MGN-617 to Typhi ISP1820 by overnight plate-mating on LB with DAP. Transconjugants were selected by growth on LB plates containing chloramphenicol without DAP. Selection for double-crossover allele replacement was obtained by *sacB* counterselection on LB agar plates without NaCl and containing 5 % sucrose (Kaniga *et al.*, 1991). Isogenic strain DEF061 contains a non-polar mutation of *taiA*. For deletion of *clyA*, the same method was used with primers clyA-BF, clyA-BR, clyA-EF and clyA-ER. The resulting isogenic strain DEF062, constructed with plasmid pSIF025, contains a non-polar mutation of *clyA*. Mutations were confirmed by PCR. Both mutants have growth curves in LB similar to that of the wild-type strain (data not shown). To complement these mutants, *taiA* and *clyA* were cloned separately into the low-copy-number vector pWSK29. This plasmid has been shown to have no deleterious effect on Typhi infection of host cells (Abromaitis *et al.*, 2005). *taiA* and *clyA* were amplified with Elongase (Invitrogen) with primers STY1499-FC, STY1499-ER and clyA-FC, clyA-ER, respectively. PCR fragments were digested with *Bam*HI and *Xba*I and ligated into pWSK29, resulting in plasmids pWSK*taiA* and pWSK*clyA*. pWSK*taiA* was transformed into the wild-type strain to investigate the effect of overexpression and into DEF061 to complement the *taiA* mutation, resulting in strains DEF074 and DEF075, respectively. To complement the *clyA* mutation, pWSK*clyA* was transformed into DEF062 to produce DEF124.

Generation of mutant strains of SPI-1 and SPI-2 T3SS. Mutations of *invA* and *ssrB* were done by using the approach described above. Primers invA-BF, invA-BR, invA-EF and invA-ER were used to generate a mutant allele for *invA*, and primers ssrB-BF, ssrB-BR, ssrB-EF and ssrB-ER for *ssrB*. These fragments were cloned into pMEG-375, and digested by *Bam*HI and *Xba*I, resulting in plasmids pSIF072 and pSIF074, respectively. Allelic exchange was performed as described above, and mutations were confirmed by PCR. Typhi strain DEF147 corresponds to an *invA* mutant and DEF149 corresponds to a *ssrB* mutant.

Epitope tagging of TaiA. Primer 1499-R-2HA was designed to contain the last 22 nt (without the stop codon) of *taiA* and two haemagglutinin (HA) tag sequences. This primer was used with STY1499-FC to PCR-amplify STY1499 with its native promoter and add two HA sequences at its 3′ end. The resulting fragment was digested with *Xba*I and *Bam*HI and ligated into pWSK29 to create

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
***S. enterica* serovar Typhi strains**		
ISP1820	Wild-type Typhi	Hone *et al.* (1991)
DEF061	ISP1820 Δ*taiA*	This study
DEF062	ISP1820 Δ*clyA*	This study
DEF074	ISP1820 (pSIF029)	This study
DEF075	DEF061 (pSIF029)	This study
DEF124	DEF062 (pSIF051)	This study
DEF130	ISP1820 (pWSK29)	This study
DEF147	ISP1820 Δ*invA*	This study
DEF149	ISP1820 Δ*ssrB*	This study
DEF150	ISP1820 (pSIF069)	This study
DEF169	DEF147 (pSIF069)	This study
DEF171	DEF149 (pSIF069)	This study
DEF429	χ8521 (pSIF069)	This study
χ8521	ISP1820 Δ*phoP*	R. Curtiss III
***E. coli* strains**		
DH5α	*supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Invitrogen
MGN-617	SM10 λpir *asd thi thr leu tonA lacY supE recA* RP4 2-Tc::Mu[λpir] Δ*asdA4*	Kaniga *et al.* (1998)
Plasmids		
pMEG-375	*sacRB mobRP4 ori*R6K; Cm^r, Ap^r	Dozois *et al.* (2003)
pWSK29	Low-copy-number cloning vector; Amp^r	Wang & Kushner (1991)
pSIF024	pMEG-375 with flanking region of *taiA* used for *taiA* deletion	This study
pSIF025	pMEG-375 with flanking region of *clyA* used for *clyA* deletion	This study
pSIF029 (pWSK*taiA*)	pWSK29 carrying *taiA*	This study
pSIF051 (pWSK*clyA*)	pWSK29 carrying *clyA*	This study
pSIF069 (p*taiA*-2HA)	pWSK29 carrying *taiA* tagged with 2HA epitope	This study
pSIF072	pMEG-375 with flanking region of *invA* used for *invA* deletion	This study
pSIF074	pMEG-375 with flanking region of *ssrB* used for *ssrB* deletion	This study

p*taiA*-2HA. This plasmid was transformed into Typhi strain ISP1820 to generate strain DEF150. Production of a 29 kDa protein detectable by anti-HA antibody was confirmed by Western blotting. p*taiA*-2HA was also transformed into the SPI-1 (DEF147), SPI-2 (DEF149) and *phoP* (χ8521) mutants, resulting in strains DEF169, DEF171 and DEF429, respectively.

Infection of human cultured macrophages. The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 (Wisent) containing 10 % (v/v) fetal calf serum (FCS) (Invitrogen), 25 mM HEPES (Wisent), 2 mM L-glutamine, 1 mM sodium pyruvate and 1 % modified Eagle's medium (MEM) non-essential amino acids (Wisent). A stock culture of these cells was maintained as monocyte-like, non-adherent cells at 37 °C in an atmosphere containing 5 % (v/v) CO_2. For macrophage infection, cells were seeded at 5×10^5 cells per well in 24-well tissue-culture dishes and differentiated by addition of 10^{-7} M phorbol 12-myristate 13-acetate for 72 h. Bacteria were grown overnight standing in LB, which corresponds to OD_{600} 0.6 ($\sim 3 \times 10^8$ c.f.u. ml^{-1}), and were then added to the cell monolayer at a m.o.i. of 10 : 1, and centrifuged for 5 min at 800 ***g*** to synchronize bacterial uptake. After incubation for 20 min at 37 °C, the infected cells were washed three times with prewarmed PBS, pH 7.4, and the infected monolayers were either lysed (t_0) from the tissue-culture dishes or incubated for 2 h with medium supplemented as above containing 100 μg gentamicin ml^{-1} (Wisent) to kill extracellular bacteria. Then, the infected monolayers were washed three times with prewarmed PBS and further incubated for an additional 46 h in the presence of fresh supplemented tissue-culture medium containing 12 μg gentamicin ml^{-1}. The infected cells were then washed three times with prewarmed PBS and the infected monolayers were lysed (t_{48}). The cells were lysed by addition of 1 ml 0.1 % (w/v) sodium deoxycholate in PBS (PBS-DOC) per well, and the number of surviving bacteria was determined as c.f.u. by plating on LB agar. The c.f.u. of the bacterial inocula were determined and the bacterial uptake was expressed as the percentage of bacteria recovered at t_0 compared with the inoculum. The survival (fold increase) corresponds to the percentage of bacteria recovered at 48 h post-infection divided by the number of bacteria at t_0. Results are expressed as the mean ± SE of the replicate experiments. The Wilcoxon signed rank test was used for statistical analysis. When indicated, the macrophages were incubated 1 h prior to infection with 1 μg cytochalasin D ml^{-1} (Sigma) to inhibit bacterial uptake, as described by Rosenshine *et al.* (1994). The addition of cytochalasin D was maintained throughout the infection.

Infection of human cultured epithelial cells. HeLa cells (ATCC CCL-2) were grown in Dulbecco's MEM (Wisent) supplemented with 10 % (v/v) heat-inactivated FCS (Wisent) and 25 mM HEPES (Wisent) (complete medium). Infection was carried out as described by Elsinghorst (1994). One day before infection, cells were seeded at 2.5×10^5 cells per well in 24-well tissue-culture plates. One hour before infection, cells were washed three times with prewarmed PBS, and fresh complete medium was added to each well. Bacteria were grown overnight in LB NaCl to OD_{600} 0.6 ($\sim 3 \times 10^8$ c.f.u. ml^{-1}) and added to each well at an m.o.i. of 20. The 24-well plates were then centrifuged at 800 ***g*** for 5 min to synchronize infection, incubated at 37 °C in 5 % (v/v) CO_2 for 90 min and rinsed three times with PBS.

Table 2. Primers used in this study

Primer	Sequence
STY1499-BF	CTGTAATGCCTCGCCACGATCCATGATTAAG
STY1499-BR	GCTCTAGAGCAAGCAGTTGAATCCAAAGGG
STY1499-EF	CGGGATCCCCAGAAGCTGGAAATACTGCCTG
STY1499-ER	CGTGGCGAGGCATTACAGCAAGCCATTTTGG
clyA-BF	GCAGCAATAGTCACGACACCACACCATTCATAA
clyA-BR	GCTCTAGAAACCGCAGATGGGGCATTAG
clyA-EF	CGGGATCCGCTATCGGGCGTTAAAAGTACACAG
clyA-ER	TGTCGTGAGTATTGCTGCGGGCGTGATTGAAGG
invA-BF	CGGGATCCCCTACAAGCATGAAATGGCAGAAC
invA-BR	AGGACAAGACTTCAATCAAGATAAGACGGCTGG
invA-EF	TGATTGAAGTCTTGTCCTCCTTACGTCTGTCG
invA-ER	GCTCTAGACGCCCAGATCCATACATCATCG
ssrB-BF	CGGGATCCAAGGCTGTTTAGGTCAAATAGGGC
ssrB-BR	CTTCGGGCGGATAAGTATGTCAGGCTCGTATGCG
ssrB-EF	ATACTTATCCGCCCGAAGAATGAGGTTAATAG
ssrB-ER	GCTCTAGATCGCCGATAGAATACGACATGG
STY1499-FC	GCTCTAGAGAGGCAACCACCAGCCCTGTC
clyA-FC	GCTCTAGATGTCTGGAGGTAATAGGTAAG
1499-R-2HA	CGGGATCCCTATTAAGCGTAGTCTGGGACGTCGTATGGGTAAAGAGCGT-AGTCTGGGACGTCGTATGGGTAAGATCTACGCAGGGTACGATTACTC
16s-F-qPCR	CGGGGAGGAAGGTGTTGTG
16s-R-qPCR	CAGCCCGGGGATTTCACATC
1499-F-qPCR	ATATCACCGATGCGGTGGGAATC
1499-R-qPCR	ACTTTCACCATTCCATCTTCCGGC
1498-F-qPCR	ACGGAAACCGAAACAACCAGATTC
1498-R-qPCR	GCGTCTTCTTACCGTGTCTTTGTTGG
pagC-F-qPCR	TTTAATGGTTGGGCCAGCCTATCG
pagC-R-qPCR	TTAAATGTCGCCTTTACCGTGCCG
prgI-F-qPCR	CAGGTAACAGAGGCGCTGGATAAA
prgI-R-qPCR	TTACCGTGTTCGATTGCGCGTTAC
sseB-F-qPCR	ATATGGCGATCATGGGAAGCTGGA
sseB-R-qPCR	TCGGTATTCCGGTTGGCGTCATTA

Cells were either lysed with 1 ml PBS-DOC to evaluate the level of adherence (t_0) or incubated for a further 90 min with complete medium containing 100 μg gentamicin ml^{-1} to kill extracellular bacteria and assess the invasion level (t_{90}). Cells were then lysed as described above. The invasion level corresponds to the number of bacteria recovered after 90 min of gentamicin treatment compared with the number of bacteria at t_0. Results are expressed as the mean ± SE of the replicate experiments. Statistical differences were assessed using the Wilcoxon signed rank test.

Cytotoxicity assay. Human cells (THP-1 or HeLa) were seeded in 24-well plates and infected as described above. After 48 h of infection for THP-1 cells and 90 min for HeLa cells, supernatants were collected and lactate dehydrogenase (LDH) release was evaluated with the Cytotox96 kit (Promega) according to manufacturer's instructions. LDH released is expressed as $100 \times [(\text{experimental release} - \text{spontaneous release})_{\text{test strain}}/(\text{experimental release} - \text{spontaneous release})_{\text{control strain}}]$, in which spontaneous release is the amount of LDH activity in the supernatant of uninfected cells. Results are expressed as the mean ± SE of the replicate experiments. Statistical differences were assessed using the Wilcoxon signed rank test.

RNA isolation, reverse transcription and real-time quantitative PCR (qPCR). RNA was isolated from bacteria in the supernatant of infection, and at 0, 8 and 24 h post-infection of human macrophages (infected as described above) by using TRIzol reagents as described by the manufacturer (Invitrogen). RNA was then precipitated with 2.5 M LiCl (Ambion) for 30 min at −20 °C, washed with ice-cold 75 % (v/v) ethanol and resuspended in diethyl pyrocarbonate-treated water. Rigorous DNase treatment was then performed to remove any trace of DNA (DNA-free kit, Ambion). Purity of extracted RNA was verified by spectrometry and absence of contaminating DNA was confirmed by real-time quantitative PCR (qPCR) with primers 16s-F and 16s-R (data not shown). A total of 50 ng RNA was reverse-transcribed by using Superscript II (Invitrogen) with 0.5 μg random hexamers (Sigma). As a negative control, a reaction without Superscript II was also included (NRT). qPCRs were performed in a Rotor-Gene 3000 thermal cycler (Corbett Research) by using the QuantiTect SYBR Green PCR kit (Qiagen), according to manufacturer's instructions. Primers used are described in Table 2. The transcriptional level of the genes of interest under each condition was normalized against a reference gene (16S rRNA) and analysed by applying the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). For each condition, reverse transcription was done three times independently, and the NRT sample was used as a negative control.

In vitro* secretion assays.** Bacteria were grown in either 5 ml LB NaCl standing or in LPM, pH 5.8, with shaking to OD_{600} 0.6. Bacteria were pelleted by centrifugation at 12 000 ***g for 5 min at 4 °C. The supernatant was collected, treated with 0.1 mM PMSF (Sigma) and

filtered through a 0.2 μm pore-size syringe filter (Fisher), and then 1.8 ml was precipitated with TCA (10 % final concentration, v/v) at 4 °C for 16 h. The TCA-insoluble fraction was collected by centrifugation, washed two times with ice-cold acetone and resuspended in an appropriate volume of SDS-PAGE loading buffer [62.5 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.05 % (w/v) β-mercaptoethanol, 0.05 (w/v) bromophenol blue] according to the OD_{600} of the original culture. When necessary, samples were neutralized with 1 μl 1.5 M Tris-HCl, pH 8.8. The bacterial pellet was also dissolved in an appropriate volume of SDS-PAGE loading buffer, according to the OD_{600} of the original culture. Proteins were separated on 10 or 15 % (w/v) SDS-polyacrylamide gels and then transferred onto PVDF membranes by using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. Membranes were blocked overnight in Tris-buffered saline containing 0.1 % (v/v) Tween 20 (TBST) and 5 % (w/v) non-fat dried milk at 4 °C. Blots were then incubated for 1 h at room temperature with either rabbit affinity-isolated anti-HA tag (1 : 5000) (Sigma), rabbit anti-GroEL (1 : 40000) (Sigma) or rabbit affinity-purified antibodies raised against recombinant SseB (1 : 2000) (Coombes *et al.*, 2004) and recombinant SopB (1 : 2500) (Coombes *et al.*, 2005) in TBST with 2.5 % (w/v) non-fat dried milk. Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used as the secondary antibody at a 1 : 5000 dilution in TBST with 2.5 % (w/v) non-fat dried milk for 1 h at room temperature. ECL plus Western blotting detection reagent (GE Healthcare) was used to detect antibody complexes. Blot images were acquired with a Typhoon Trio scanner using the ECL+ setting (GE Healthcare).

RESULTS

Presence of *clyA* and *taiA* in bacterial genomes

BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) analysis using the sequence encoding *clyA* and *taiA* genes reveals that this gene cluster is present in *S. enterica* serovar Typhi, Paratyphi A, Javiana and Schwarzengrund, but absent in other sequenced serovars, including Typhimurium (Fig. 1a), and absent in other microbial genomes. This gene cluster possesses a GC content of 40 %, which is relatively low compared with the average of 52 % for the Typhi genome (Parkhill *et al.*, 2001). By comparative genomic hybridization, serovars Montevideo, Oranienfoburg, Sendai and Muenchen were also shown to possess these genes, although only one strain per serovar was tested (Porwollik *et al.*, 2004). The presence of *clyA*, by itself, has been observed in *E. coli*, in both pathogenic and K-12 strains (Ludwig *et al.*, 2004). BLAST analysis with the nucleotide sequence revealed that *clyA* is also present in *Shigella flexneri* serotypes 2a and 5. However, *taiA* was absent from *E. coli*, and BLAST analysis using the nucleotide sequence did not identify any homologous genes. However, BLAST analysis at the protein level using the coliBASE database (http://colibase.bham.ac.uk/) revealed homology to a hypothetical protein in *Yersinia* spp. Putative subcellular localization of TaiA using PSORTb was unable to predict a subcellular localization, and no putative signal peptide for secretion was identified (Gardy *et al.*, 2005).

clyA and *taiA* are organized in an operon

Possible promoters for *taiA* and *clyA* were searched for using NNPP version 2.2 with the prokaryote settings (http://www.fruitfly.org/seq_tools/promoter.html). A putative promoter sequence was detected 200 bp upstream of *taiA*, but none was found upstream of *clyA*. This suggested that *clyA* was transcribed from the *taiA* promoter and that they might be organized in an operon. The transcriptional linkage was verified by RT-PCR. PCR was performed on cDNA, and a 1.2 kb fragment which encompassed *taiA* and *clyA* was amplified with primers 1499-F and 1498-R (Fig. 1b). Therefore, *taiA* and *clyA* are co-transcribed and constitute an operon.

clyA and *taiA* are expressed in human macrophages

Transcriptomic analysis of Typhi infecting human cultured macrophages (THP-1) has shown that *taiA* and *clyA* are induced during infection (Faucher *et al.*, 2006). To confirm this finding, qPCR was performed on RNA samples from bacteria present in the supernatant of infection and from infected macrophages at 0, 8 and 24 h post-infection (Fig. 1c). The supernatant sample contained RNA from bacteria that were not associated with macrophages and was used as the control condition, as previously described for the microarray experiment (Faucher *et al.*, 2006). The 0 h time point contains RNA from bacteria associated with macrophages, either extracellularly or intracellularly. Both genes seemed to be strongly induced when the bacteria were associated with macrophages (0 h), and even more at later time points during the infection. The lower expression of *clyA* compared with *taiA* might be due to a lower mRNA stability of *clyA*, as differential mRNA stability in a polycistronic operon has been shown to be a common mechanism of post-transcriptional regulation in bacteria (Grunberg-Manago, 1999). Nonetheless, both genes have the same expression pattern, which corroborates the results indicating that these genes are co-transcribed.

TaiA, but not ClyA, is involved in macrophage uptake

Upregulation of *clyA* and *taiA* expression during early association of Typhi with human macrophages suggests that they have an effect on macrophage uptake. To test this hypothesis, mutant strains harbouring a non-polar deletion of *taiA* or *clyA* were constructed, and their contribution to uptake by human cultured macrophages (THP-1) was investigated using a gentamicin protection assay. The *clyA* deletion did not affect bacterial uptake significantly. However, deletion of *taiA* reduced bacterial association or uptake by human macrophages to 60 % ($P<0.005$) of the wild-type value (Fig. 2a). Complementation of the *taiA* mutant with a wild-type copy of the gene on a low-copy-number plasmid restored the wild-type phenotype. In order to differentiate between bacterial association and

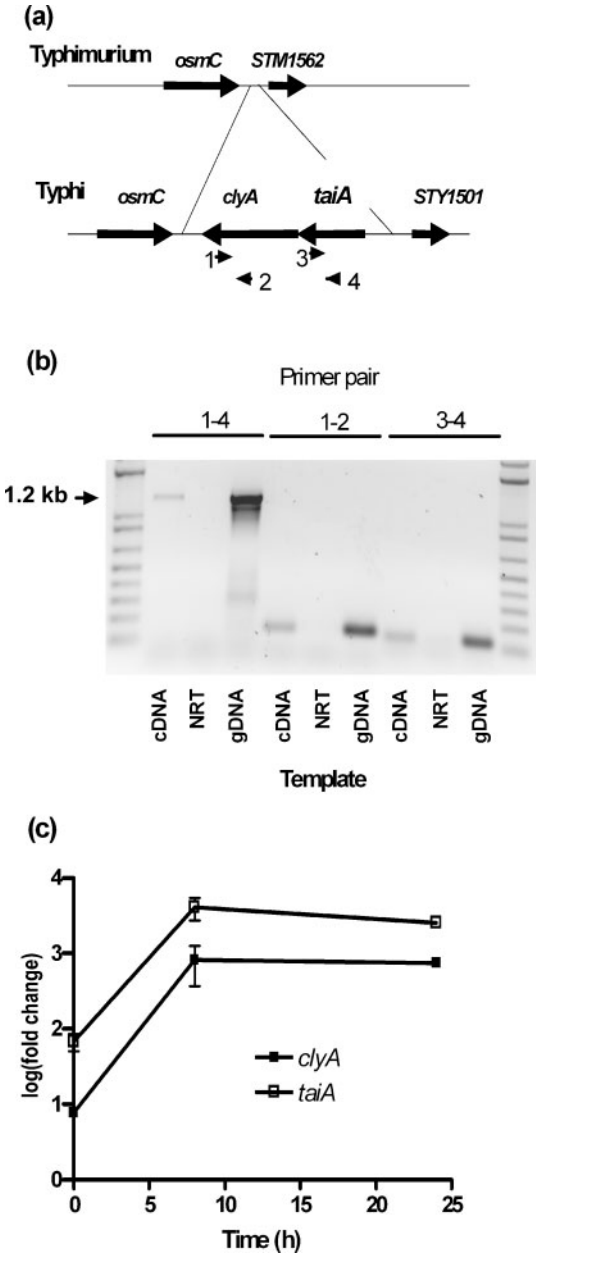

Fig. 1. Genetic organization and expression of *taiA* and *clyA*. (a) DNA region encoding *taiA* and *clyA* in Typhi and the corresponding DNA region in Typhimurium. (b) The transcription unit was analysed by RT-PCR. Primers used are depicted in (a): 1, 1498-R-; 2, 1498-F-; 3, 1499-R-; 4, 1499-F-qPCR. NRT, no reverse transcriptase (negative control). (c) qPCR validation of expression of *taiA* and *clyA* inside human macrophages, compared with the supernatant of infection.

phagocytosis, macrophages were pretreated with cytochalasin D before infection. Cytochalasin D is an inhibitor of host cell cytoskeletal function and blocks bacterial uptake by macrophages (Rosenshine *et al.*, 1994). In the presence of cytochalasin D, only 3–4 % of the initial bacterial inoculum was associated with macrophages, and the level of association of the Δ*taiA* strain was similar to that of the wild-type (Fig. 2a). Taken together, these results suggest that TaiA, but not ClyA, is involved in increasing bacterial uptake by host macrophages by a mechanism independent of bacterial adhesion. Because Δ*clyA* and Δ*taiA* strains have different phenotypes, it is unlikely that the effect observed for the Δ*taiA* strain was due to a polar effect of the *taiA* mutation on *clyA* expression.

ClyA, but not TaiA, affects survival inside macrophages

The contribution of *clyA* and *taiA* to Typhi survival in human cultured macrophages (THP-1) was investigated using the gentamicin protection assay. The *taiA* deletion did not affect survival significantly, but *clyA* deletion enhanced survival of Typhi in human macrophages by 40 % (P=0.02) of the wild-type phenotype, after 48 h of infection (Fig. 2b). Complementation of the mutant strain with a low-copy-number plasmid harbouring *clyA* decreased survival significantly (P=0.03). In order to investigate the effect of ClyA production inside macrophages, a cytotoxicity assay was performed by monitoring the release of LDH, a cytolysis indicator. Macrophages infected with the Δ*clyA* strain or the complemented mutant strain released the same amount of LDH as the macrophages infected by the wild-type strain (Fig. 2c).

Involvement of *taiA* and *clyA* during infection of human epithelial cells

Since TaiA seems to be involved in invasion of macrophages, and a published study has reported that purified *E. coli* ClyA is cytotoxic to epithelial cells (Wai *et al.*, 2003), we investigated the role of TaiA and ClyA during invasion of epithelial cells. Surprisingly, the level of invasion of epithelial cells was similar for the Δ*taiA* strain and the wild-type strain (Fig. 3a). Interestingly, when an additional copy of *taiA* was added to the wild-type strain, a much higher level of invasion was observed, compared with the wild-type strain harbouring the empty vector (Fig. 4a). The Δ*clyA* strain was two times more invasive (P=0.03) than the wild-type strain (Fig. 3a). In addition, the Δ*clyA* strain was 25 % less cytotoxic (P=0.015) towards epithelial cells than the wild-type strain (Fig. 3b). Complementation restored the wild-type phenotype.

TaiA enhances *E. coli* invasion of HeLa

As TaiA was involved in Typhi uptake by host cells, we next investigated the effect of *taiA* addition to non-invasive *E. coli* during interaction with epithelial cells. Therefore, *E. coli* DH5α was transformed with pWSK*taiA* and pWSK29 and invasion assays were performed. Similarly to the results obtained with Typhi, *E. coli* harbouring pWSK*taiA* was two times more invasive (P=0.03) than *E. coli* harbouring the empty vector (Fig. 4b). No difference was observed for the initial adhesion levels (data not shown).

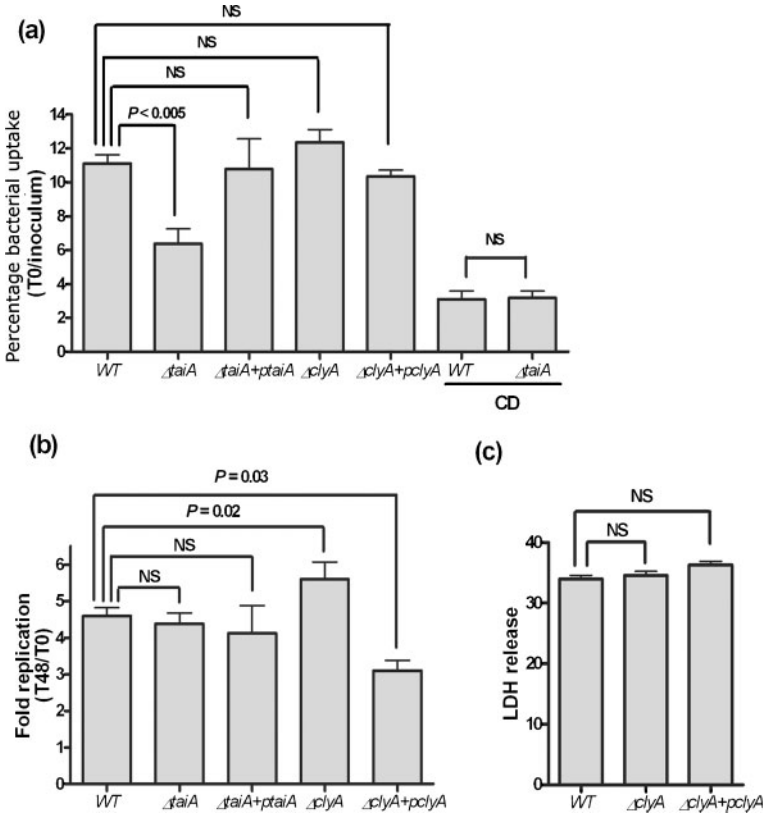

Fig. 2. Interaction of Δ*taiA* and Δ*clyA* strains with human macrophages. (a) Typhi uptake by macrophages was investigated by comparing the number of bacteria at 0 h with the number of bacteria added to the well. The effect of *taiA* mutation on bacterial uptake was confirmed by treatment of macrophages with cytochalasin D (CD), an inhibitor of actin polymerization. (b) Typhi replication inside human macrophages was investigated by comparing the number of bacteria at 48 h post-infection with the number of bacteria at 0 h (fold increase). (c) Cytotoxicity of Δ*clyA* was investigated by quantifying LDH release at 48 h post-infection. Results are shown as a percentage of the control value for each replicate; see text for details. Experiments were replicated at least three times independently; NS, not significant.

TaiA is a novel secreted protein

To allow detection of TaiA by Western blotting, the protein was tagged at its C-terminal end with two HA epitopes and cloned in a low-copy-number vector with its native promoter. The HA tag has been proven useful for studying protein secretion, and C-terminal tags are usually well tolerated (Uzzau *et al.*, 2001). The presence of TaiA-2HA in the supernatant of bacteria grown in conditions known to induce expression of genes involved in invasion of host

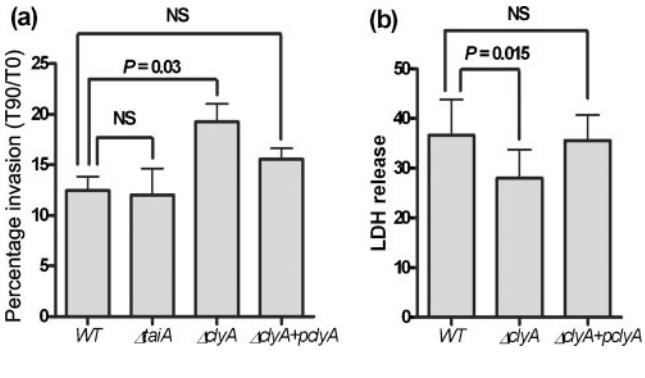

Fig. 3. Interaction of Δ*taiA* and Δ*clyA* strains with human epithelial cells. (a) Typhi invasion of epithelial cells was investigated by comparing the number of bacteria at 90 min with the number of bacteria at 0 h. (b) Cytotoxicity was investigated by quantifying LDH release at 90 min post-infection. Results are shown as a percentage of the control value for each replicate; see text for details. Experiments were replicated at least three times independently; NS, not significant.

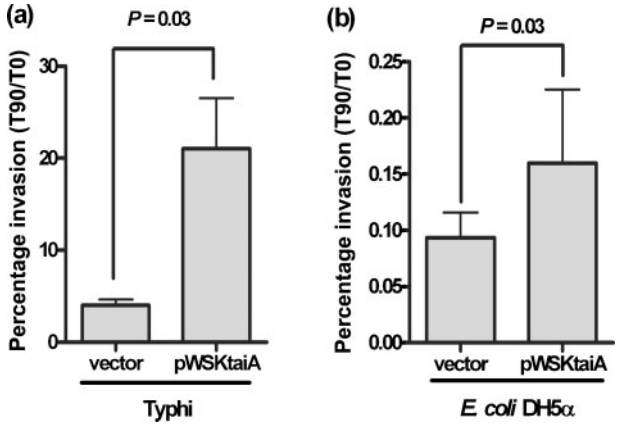

Fig. 4. Effect of *taiA* overexpression during interaction with epithelial cells. HeLa cells were infected with Typhi (a) or *E. coli* (b) harbouring the empty vector (pWSK29) or pWSK*taiA*, and the invasion rate was determined. Results are shown as a percentage of the control value for each replicate; see text for details. Experiments were replicated at least three times independently.

cells was investigated (LB NaCl) (Lostroh & Lee, 2001). A 29 kDa protein was detected by anti-HA antibodies in the supernatant fraction and in the pellet fraction of the strain harbouring p*taiA*-2HA, but not in those of the strain harbouring the vector alone (Fig. 5a, lanes 1 and 2). The cytoplasmic GroEL protein was not detected in the supernatant fraction, indicating that it is unlikely that TaiA-2HA was detected because of cytoplasmic leakage. Therefore, TaiA is a novel secreted protein of Typhi involved in uptake by host cells.

TaiA is secreted independently of SPI-1 or SPI-2 T3SS

Because TaiA seems to be involved in early interaction with human macrophages and because it is secreted in SPI-1-inducing conditions (LB NaCl), we investigated whether TaiA secretion was mediated by the SPI-1 T3SS. This was achieved by monitoring secretion of TaiA-2HA in a Δ*invA* strain, which is unable to assemble a functional SPI-1 T3SS (Sukhan *et al.*, 2001). As expected, the Typhi Δ*invA* strain was less able to invade human epithelial cells (data not shown) and was not able to secrete SopB (Fig. 5a, lane 3). TaiA-2HA was still secreted by a Δ*invA* strain, indicating that its secretion in SPI-1-inducing media was independent of SPI-1 T3SS (Fig. 5a, lane 3).

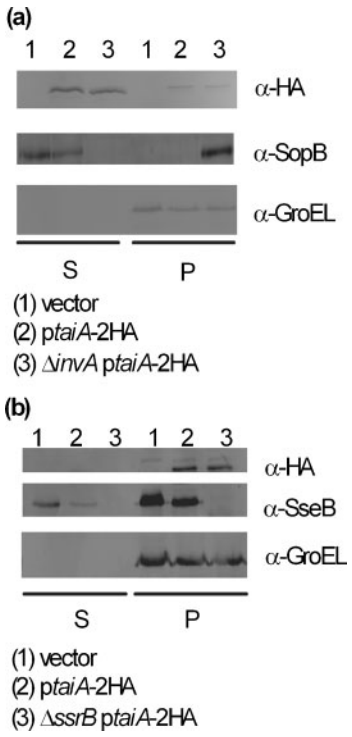

Fig. 5. TaiA is secreted independently of both T3SSs. Bacteria expressing TaiA with a double HA-tag at its C-terminal were used to investigate TaiA secretion. Bacteria harbouring the vector alone were used as a negative control. Bacteria were grown in (a) LB NaCl overnight standing (SPI-1-inducing condition) or (b) LPM, pH 5.8, aerobically (SPI-2-inducing condition). Proteins in the supernatant (S) and bacterial pellet (P) were harvested and subjected to Western blotting. Isogenic Δ*invA* and Δ*ssrB* strains were used as negative controls for SPI-1 and SPI-2 T3SS-mediated secretion, respectively. See text for details.

Since TaiA was not secreted by SPI-1 T3SS and because it was involved in Typhi interaction with human macrophages, it may be secreted by SPI-2 T3SS, which is used to translocate bacterial effectors into host macrophages; therefore, the secretion of TaiA by the SPI-2 T3SS was investigated. Under SPI-2-inducing conditions, a 29 kDa protein, detected by anti-HA tag antibodies, was produced by bacteria harbouring p*taiA*-2HA (Fig. 5b). However, this protein was detected only in the bacterial pellet and not in the culture supernatant (Fig. 5b). As a control for SPI-2 secretion, SseB was detected in the culture supernatant of Typhi strains harbouring the vector alone or p*taiA*-2HA, but was absent in the Δ*ssrB* strain (Fig. 5b, lane 3). GroEL was used as a control for bacterial cytoplasm leakage and was not detected in the culture supernatant. Our data show that TaiA is not secreted under SPI-2-inducing conditions, and it is therefore unlikely that TaiA is secreted by the SPI-2 T3SS.

taiA and *clyA* expression is regulated by PhoP

The PhoPQ two-component system regulates expression of many intracellular genes (Bijlsma & Groisman, 2005; Groisman, 2001). Since *taiA* and *clyA* are involved during infection of human macrophages, this raises the possibility that PhoPQ regulates their expression. Therefore, we compared the expression of *taiA* and *clyA* in the wild-type strain and in a *phoP* mutant in growth conditions known to activate (low magnesium) or inactivate PhoP (high magnesium) (Garcia Vescovi *et al.*, 1996). In the wild-type strain, when PhoP was activated, *taiA* and *clyA* were 100- and 35-fold more expressed, respectively (Fig. 6a). However, their expression was much lower in the *phoP* mutant (Fig. 6a). Under these growth conditions, *taiA* and *clyA* expression was similar to that of *pagC*, a PhoPQ-activated gene. TaiA-2HA was highly produced under PhoP-activating conditions, completely abolished in the Δ*phoP* strain and barely detectable when grown in conditions in which PhoP is inactive, as visualized by Western blotting (data not shown). However, TaiA-2HA was not secreted under these conditions. We then investigated by qPCR the expression of *taiA* and *clyA* in the wild-type strain and in a *phoP* mutant during infection of THP-1 macrophages 24 h post-infection. *pagC* was downregulated almost 100-fold in the *phoP* mutant, validating this method for investigating gene regulation by PhoP inside macrophages (Fig. 6b). A 10-fold reduction of both *taiA* and *clyA* expression was observed in the *phoP* mutant (Fig. 6b). It is well known that PhoPQ also regulates the SPI-2 T3SS, which may explain why *taiA* and *clyA* have intracellular expression profiles similar to those of some SPI-2 T3SS genes. However, induction of SPI-2

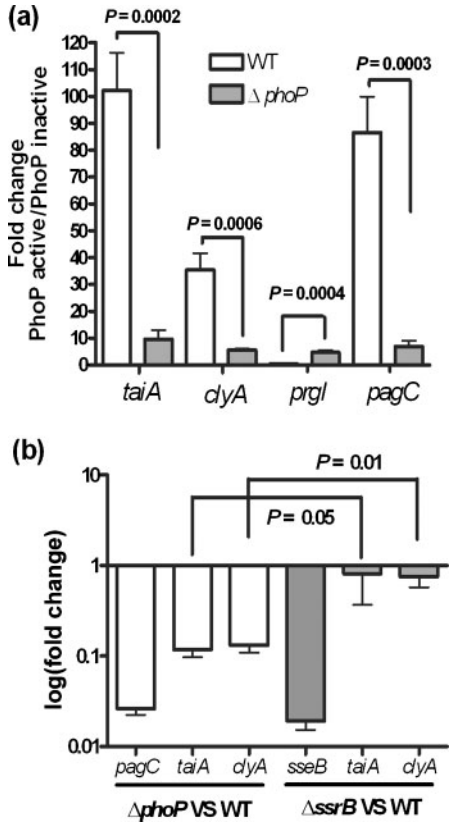

Fig. 6. PhoP regulates expression of *taiA* and *clyA*. (a) Real-time qPCR was used to compare expression of *taiA* and *clyA* in conditions in which PhoP is known to be active (low concentration of magnesium, low pH) or inactive (high concentration of magnesium, pH 7.4) in the wild-type strain and in a Δ*phoP* (DEF429) strain. *pagC* and *prgI* are known to be activated and repressed, respectively, by the active form of PhoP, and were used as controls. (b) Real-Time qPCR was used to compare the expression of *taiA* and *clyA* in the wild-type strain and in a Δ*phoP* (DEF429) or Δ*ssrB* (DEF149) strain at 24 h post-infection of human macrophages. The fold change represents the level of normalized expression in the mutant strain compared with the wild-type strain. *pagC* and *sseB* were used as controls for PhoP- and SsrB-dependent expression, respectively. See Methods for details.

T3SS structural and effector genes require the *ssrA/ssrB* two-component system, which is also controlled by the PhoPQ system (Hensel *et al.*, 1998; Waterman & Holden, 2003). To investigate a possible regulation of *taiA* and *clyA* by SsrB, we compared expression of these genes in the wild-type and in a Δ*ssrB* isogenic strain, 24 h post-infection of human macrophages. Expression of *taiA* and *clyA* in a Δ*ssrB* strain was similar to their expression level in the wild-type strain background (Fig. 6b). As expected, the SPI-2-encoded effector *sseB* was strongly repressed in a Δ*ssrB* strain. Relative expression values for *taiA* and *clyA* between the Δ*phoP* and Δ*ssrB* strains were significantly different ($P \leqslant 0.05$). Thus, *taiA* and *clyA* are part of the PhoP regulon but not the SsrB regulon.

DISCUSSION

The goal of this study was to assess the implication of a gene cluster, composed of two ORFs named *taiA* and *clyA* that were upregulated during macrophage infection, during Typhi interaction with host cells. We first demonstrated by transcriptional analysis that *taiA* and *clyA* are co-transcribed, since a 1.2 kb cDNA overlapping both genes was amplified by PCR (Fig. 1b). Moreover, both genes show the same induction profiles during infection of human macrophages (Fig. 1c). The expression profile obtained by qPCR correlates well with the results obtained in the transcriptomic study of Typhi inside human macrophages (Faucher *et al.*, 2006).

Typhi ClyA contributed to cytotoxicity in epithelial cells (Fig. 3b), as has been shown for *E. coli* ClyA (Wai *et al.*, 2003). At the intestinal phase of the infection, ClyA might be useful to lyse epithelial cells to allow deep tissue infection. However, ClyA reduced long-term survival (48 h post-infection) of Typhi inside human macrophages, because the mutant strain showed an increased survival rate, without affecting cytotoxicity (Fig. 2b, c). The mechanisms underlying differences in the interaction of ClyA with epithelial cells and macrophages are currently unknown and will necessitate more tests using different epithelial and macrophage cell lines. ClyA insertion into the SCV membrane could create a pore that could alter the SCV content, which may in turn affect the survival of Typhi. The ClyA pore is at least 35 Å (0.35 nm) wide, which is sufficiently large to allow passage of small compounds such as maltose (Oscarsson *et al.*, 2002; Tzokov *et al.*, 2006). One may therefore hypothesize that the formation of pores in the SCV by ClyA affects the concentration of ions and small molecules inside the SCV. This may occur either by leakage from the host cell cytoplasm into the SCV or by leakage in the opposite direction. We can also hypothesize that the cytolysin is secreted from the intracellular compartment of infected cells, and will affect adjacent epithelial cells, as shown for the typhoid toxin CdtB (Spano *et al.*, 2008).

Because host macrophages are the reservoir of *Salmonella* during systemic infection (Richter-Dahlfors *et al.*, 1997), expression of *clyA* by Typhi inside human macrophages may lead to reduced bacterial growth and persistent infection of human macrophages during the systemic phase of infection. Growth control inside host cells by intracellular pathogens is a new concept, and some mechanisms have been reported (Tierrez & Garcia-del Portillo, 2005). SciS, a Typhimurium homologue of *Legionella pneumophila* IcmF, has been shown to reduce intracellular growth in macrophages. Interestingly, loss of *sciS* attenuates virulence of Typhimurium in mice (Parsons & Heffron, 2005). Controlled growth in host cells has also been linked to chronic infection (Monack *et al.*, 2004; Tierrez & Garcia-del Portillo, 2005). This growth control might explain the longer incubation period of Typhi with respect to Typhimurium.

As macrophages are the reservoir of *Salmonella*, increasing bacterial uptake by these cells may increase the probability of establishing a systemic infection. This function might be mediated in part by TaiA, because deletion of *taiA* reduced macrophage uptake (Fig. 2a). Reduction in bacterial uptake does not seem to be caused by a reduction in bacterial adhesion or association with human macrophages, since there was no difference in cell association between the wild-type and the Δ*taiA* strain following uptake inhibition by cytochalasin D treatment (Fig. 2a). Therefore, TaiA seems to increase macrophage phagocytic activity. This activity seems restricted to macrophages, since the *taiA* deletion did not impair invasion of epithelial cells (Fig. 3a), but additional copies of *taiA* enhanced the invasion rate (Fig. 4). Nevertheless, our results indicate that TaiA is a novel invasin of Typhi.

Production of TaiA in cell pellets was observed in SPI-1-, SPI-2- and PhoP-inducing media, but secretion of TaiA was only detected under SPI-1-inducing conditions. TaiA secretion was found to be independent of both T3SSs by Western blotting. It was surprising to note secretion of TaiA in SPI-1-inducing conditions, because these conditions are not usually associated with functions involved in interaction with host macrophages. However, there are a number of studies implicating the SPI-1 T3SS during infection of macrophages, and SPI-1-translocated effectors are involved in *S. enterica* interaction with macrophages. For example, the SPI-1 T3SS causes early macrophages apoptosis, a function attributed to SipB (Chen *et al.*, 1996; Fink & Cookson, 2006; Hersh *et al.*, 1999; Lundberg *et al.*, 1999). These studies suggest that SPI-1 T3SS-inducing conditions exist during early interaction with human macrophages. However, our data do not rule out secretion of TaiA under other conditions. Moreover, differential secretion of TaiA suggests regulation at the post-transcriptional level by an unknown mechanism that will require more investigation. Detection of TaiA in the supernatant fraction or the cell fraction at the initial time point of macrophage infection (t_0) by Western blotting was unsuccessful, probably because the TaiA concentration was too low (data not shown). Nevertheless, TaiA is a novel secreted protein independent of both T3SS and, possibly, of the Sec pathway, because no signal peptide was detected in the N-terminal portion of the protein. Because ClyA is exported by OMVs (Tzokov *et al.*, 2006; Wai *et al.*, 2003), one may hypothesize that TaiA also uses this export mechanism and that these proteins interact together in the OMVs. These possibilities are currently under investigation.

The involvement of *taiA* and *clyA* during interaction of Typhi with human macrophages prompted us to investigate a possible regulation by the PhoPQ two-component system, which regulates a number of virulence factors important for *Salmonella* survival inside human macrophages (Bijlsma & Groisman, 2005; Groisman, 2001). Expression of *taiA* and *clyA* was higher under PhoP-activating conditions and reduced in a *phoP* mutant. Similar results were obtained during infection of human macrophages, where both genes were regulated by PhoP (Fig. 6). To rule out a possible regulation by the downstream two-component system SsrA/SsrB, qPCR analysis was also performed on an *ssrB* mutant strain during infection. However, SsrB does not seem to be involved in regulation of *taiA* and *clyA* (Fig. 6). Our results show that *taiA* and *clyA* are novel members of the PhoP regulon. Regulation of the *taiA* invasin by PhoP may seem to contradict its well-accepted function as a regulator of virulence genes involved in intracellular survival, such as the SPI-2 T3SS. However, using LB broth, a condition not usually recognized to support expression of genes involved in intracellular survival, it has been shown that *pag* genes, controlled by PhoP, and some SPI-2 effectors are expressed (Belden & Miller, 1994; Bustamante *et al.*, 2008). Moreover, it was recently shown that two genes encoded on SPI-1 (*orgBC*) are activated by PhoP during growth in LB NaCl, a condition known to induce SPI-1 genes (Aguirre *et al.*, 2006). Therefore, our finding that PhoP regulates *taiA*, which is secreted under SPI-1-inducing conditions, suggests a double control of expression that may reflect a sequential requirement for this protein. The TaiA invasin will be expressed and accumulated under PhoP-activated conditions and then secreted under SPI-1-inducing conditions. However, we cannot rule out that the effect of PhoP is indirect, and we are currently investigating in more detail the mechanism of regulation. It has recently been shown that ClyA is regulated by SlyA in Typhi (von Rhein *et al.*, 2009). SlyA has been shown to be induced via the PhoPQ two-component system following internalization of bacteria by macrophages (Buchmeier *et al.*, 1997; Norte *et al.*, 2003; Shi *et al.*, 2004), and it has been shown that SlyA enhances overall transcription of PhoP-activated loci (Song *et al.*, 2008). Interestingly, a large number of SlyA-dependent genes are also controlled by the PhoPQ system (Navarre *et al.*, 2005). We hypothesize that the *taiA–clyA* gene cluster is also under co-regulation by SlyA and PhoP. Some PhoP-regulated genes are conserved among enterobacteria and others are specific to *Salmonella* (Lejona *et al.*, 2003). *taiA* and *clyA* represent PhoP-regulated genes that are specific to a limited number of *Salmonella* serovars.

This study shows that *taiA* and *clyA* are co-regulated by PhoP and seem to have complementary functions. TaiA is a novel secreted invasin, which increases bacterial uptake by human macrophages, and ClyA reduces bacterial growth within these cells, which might result in an increased use of macrophages as a sheltered environment. This in turn may promote persistent infection of the host, which is a key feature of typhoid fever.

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ANNEXE II : Résultats supplémentaires

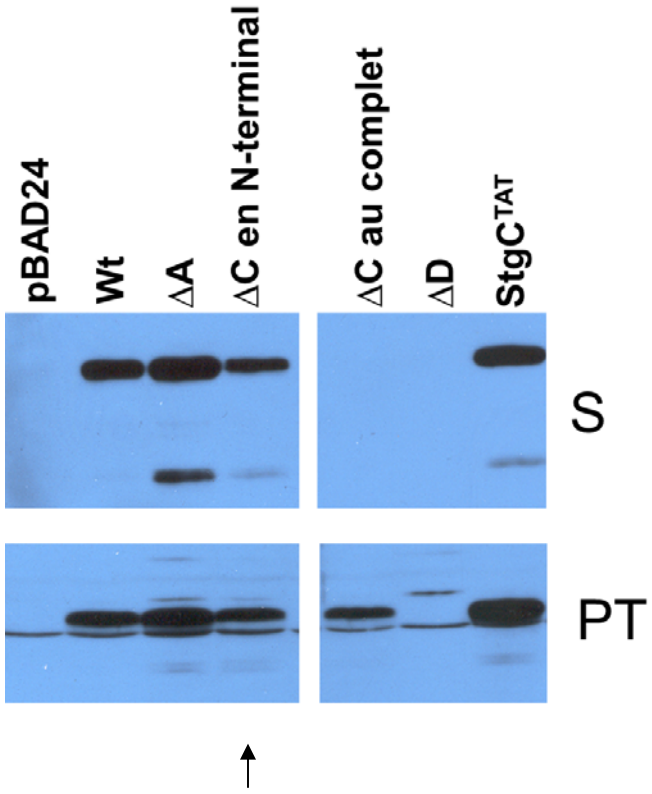

Figure S1. Exportation à la surface de StgD, suite à la délétion de résidus en N-terminal de StgC. Western blot effectué avec l'anti-StgD sur la souche ORN172 possédant différentes versions de l'opéron *stg* clonées dans le vecteur pBAD24. Wt représente l'opéron sauvage renfermant le pseudogène *stgC*, la 3^{e} ligne montre le mutant de la sous-unité majeure StgA. La délétion des résidus 2 à 202 en N-terminal de StgC permet l'exportation de StgD à la surface des bactéries (puit 4, montré par une flèche). L'absence totale de *stgC* séquestre StgD à l'intérieur des bactéries (puit 5) et la mutation complète de *stgD* démontre la spécificité de l'anticorps. La modification du codon d'arrêt pour un codon insérant une tyrosine est représentée dans le dernier puit. S signifie protéines récoltées dans les surnageants d'extraction de protéines de surface, alors que PT montre les protéines bactériennes totales. Le puit du centre a été enlevé de l'image originale.

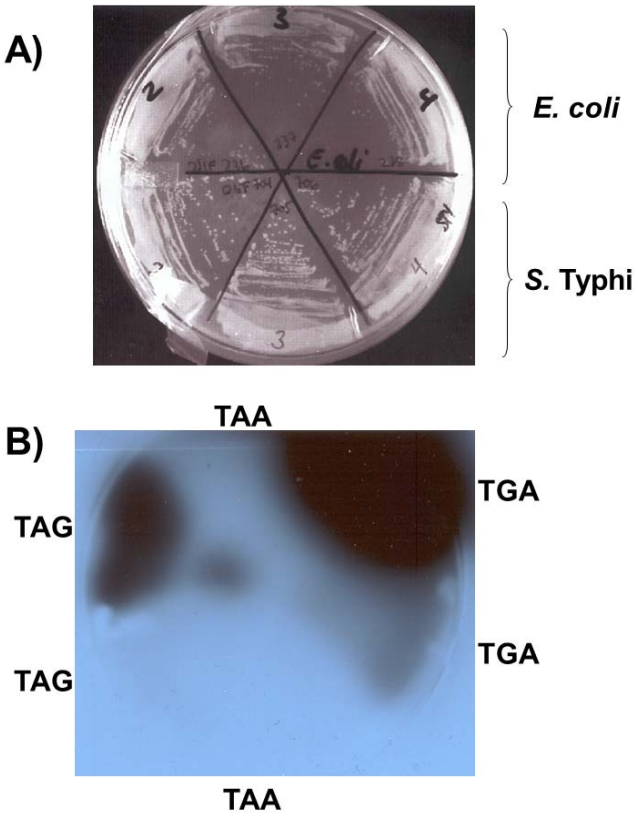

Figure S2. Suppression des trois types de codons d'arrêt chez *E. coli* et *S.* Typhi. A) Croissance sur gélose LB à 37ºC d'*E. coli* et *S.* Typhi renfermant les plasmides pLUX2, pLUX3 et pLUX4 renfermant chaque type de codon d'arrêt au 13^{e} codon de la luciférase (223). B) Un film a été laissé 1 heure sur la gélose dans le noir en présence de n-décylaldéhyde. La gélose est représentée dans le même sens qu'en A (*E. coli* en haut, *S.* Typhi en bas). Les trois types de codons d'arrêts sont identifiés. Une coloration foncée est due à l'émission de luciférase, donc indique le niveau de suppression des codons d'arrêts.

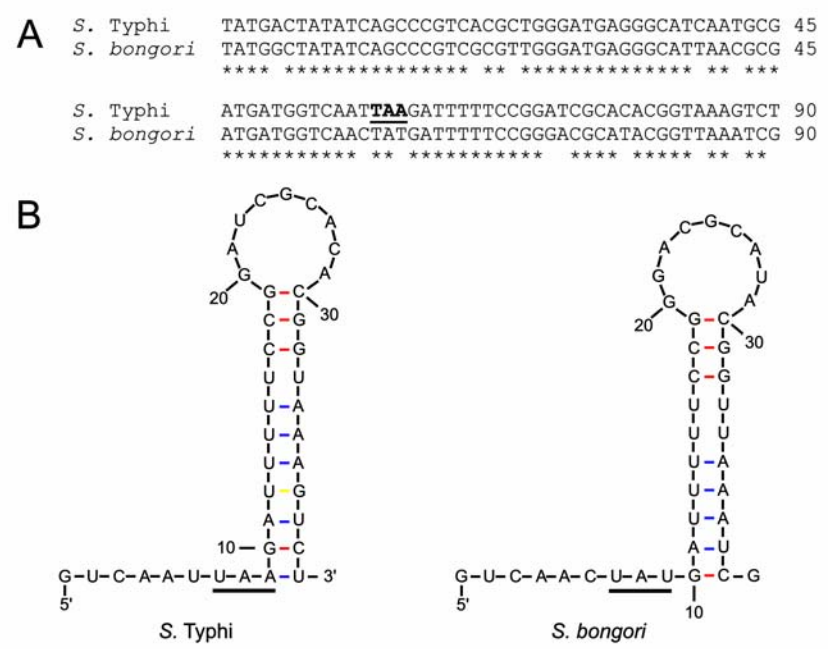

Figure S3. Comparaison entre *stgC* de la souche ISP1820 de *S.* Typhi et l'orthologue retrouvé chez *S. bongori*. A) Alignement de 90 nucléotides retrouvés dans le gène *stgC* de *S.* Typhi et de son orthologue chez *S. bongori*. La séquence représentée correspond à ce qui a été introduit dans le vecteur pXG-10 (voir Chapitre 3), soit 19 codons en amont du codon d'arrêt prématuré TAA et 10 codons en aval. Le codon d'arrêt prématuré TAA de *stgC* de la souche ISP1820 de *S.* Typhi est souligné et mis en gras. Chez *S. bongori*, un codon TAT, correspondant à l'insertion d'une tyrosine dans la protéine, est retrouvé au codon correspondant. B) Comparaison des structures en tige-boucle prédites par Mfold retrouvées dans l'ARNm *stgC* de *S.* Typhi (à gauche) et son orthologue chez *S. bongori* (à droite). Le codon d'arrêt prématuré TAA chez *S.* Typhi est souligné, ainsi que le codon correspond chez *S. bongori*. Les liaisons fortes entre 2 nucléotides sont indiquées en rouge, les liaisons de moyenne affinité en bleu et les liaisons de faible affinité en jaune.