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Identification et caractérisation de gènes impliqués dans la virulence de *Salmonella typhi* suite à une analyse globale par biopuces de l'infection de macrophages humains en culture.

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Cette thèse intitulée :

Identification et caractérisation de gènes impliqués dans la virulence de *Salmonella typhi* suite à une analyse globale par biopuces de l'infection de macrophages humains en culture.

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RÉSUMÉ

Salmonella enterica est une bactérie pathogène causant une variété de maladies chez l'homme et l'animal. Le sérovar Typhi est l'agent étiologique de l'infection systémique appelée fièvre typhoïde qui cause plus de 21 millions de nouveaux cas par année et plus de 200 000 morts. Typhi est restreint à l'homme et il n'existe pas de modèle animal pour l'étude de ses mécanismes de virulence. Pour cette raison, la majorité des connaissances sur Typhi ont été extrapolées d'études utilisant un sérovar apparenté, Typhimurium, dans un modèle murin de fièvre typhoïde. Par contre, Typhimurium cause chez l'homme une infection localisée, ce qui laisse supposer que Typhi possède des facteurs de virulence spécifiques qui lui permettent d'infecter l'homme de façon systémique. La survie de *Salmonella* dans les macrophages est déterminante pour causer une infection systémique. L'hypothèse de cette thèse est que les gènes de Typhi exprimés dans les macrophages sont impliqués dans sa pathogenèse. Les objectifs de cette thèse sont donc l'identification et la caractérisation de gènes uniques à Typhi exprimés dans les macrophages. Pour l'identification, nous avons d'abord utilisé l'approche « Selective Capture of Transcribed Sequences » (SCOTS). Cette méthode a permis d'identifier 36 gènes uniques à Typhi (absent dans le génome de Typhimurium) exprimés dans les macrophages, dont l'opéron fimbriaire *stg*. Nous avons ensuite utilisé SCOTS en conjonction avec les biopuces pour obtenir le profil d'expression global des gènes de Typhi dans les macrophages humains comparés au surnageant d'infection. Cette deuxième approche a aussi permis d'identifier 117 gènes exprimés de façon constitutive durant l'infection de macrophages dont 19 gènes uniques

à Typhi incluant *clyA* et *STY1499*. La majorité des facteurs de virulence extrapolés du modèle murin de la fièvre typhoïde ont aussi été identifiés pendant l'infection de macrophages humains par Typhi. Il s'est avéré que Typhi exprime le système de sécrétion de type trois impliqué dans la survie dans les macrophages et plusieurs gènes impliqués dans la résistance aux peptides antimicrobiens. Par la suite, nous avons caractérisé et déterminé l'implication de l'opéron fimbriaire *stg*, de *clyA* et de *STY1499* dans la pathogenèse de Typhi. Il s'est avéré que *stg* augmente l'adhérence de Typhi aux cellules épithéliales. Par contre *stg* semble restreindre la phagocytose de Typhi par les macrophages humains. ClyA est cytotoxique pour les cellules épithéliales, mais pas pour les macrophages. Par contre, ClyA semble réduire la survie de Typhi dans les macrophages. Ceci peut être important pour contrôler la multiplication de Typhi et permettre l'établissement d'une infection systémique. STY1499 est une nouvelle protéine sécrétée par Typhi, indépendamment des deux systèmes de sécrétion de type trois. STY1499 semble être impliqué dans l'invasion des cellules phagocytaires par Typhi, mais pas dans l'invasion des cellules épithéliales. STY1499 a donc été renommé TaiA pour « Typhi associated invasin A ». En conclusion, la technique SCOTS, seule ou en conjonction avec les biopuces, a identifié des gènes uniques à Typhi impliqués dans l'interaction avec les cellules humaines. Les travaux de cette thèse ont permis de mieux comprendre les mécanismes de la pathogenèse de Typhi et pourrait permettre le développement de souches atténuées utilisables comme vaccin ou comme vecteur d'antigène.

Mots clés : *Salmonella enterica* sérovar Typhi, fièvre typhoïde, infection systémique, spécificité d'hôte, « Selective Capture Of Transcribed Sequences » (SCOTS), biopuces, facteurs de virulence, fimbriae, hémolysine, invasine

ABSTRACT

Salmonella enterica is a bacterial pathogen causing a variety of diseases in humans and animals. The serovar Typhi is the etiologic agent of the typhoid fever, a systemic infection affecting more than 21 million people each year and killing more than 200,000. Typhi is a human-restricted pathogen and there are no animal models for the study of its pathogenesis. Most of the data on Typhi has therefore been extrapolated from studies using serovar Typhimurium in a murine model of typhoid fever. However, Typhimurium only causes a localised infection in humans, which suggests that Typhi possesses specific virulence factors permitting systemic infection in humans. It has been shown that *Salmonella*'s survival in macrophages is a major determinant in its capacity to cause systemic infections. The hypothesis of this thesis is that Typhi's genes expressed in macrophages are involved in its pathogenesis. The objectives of this thesis are therefore the identification and characterisation of genes unique to Typhi expressed in macrophages. For the identification, we first used the Selective Capture of Transcribed Sequences (SCOTS) approach. This method has permitted the identification of 36 genes unique to Typhi (absent from the Typhimurium genome) expressed in macrophages, one of which is the *stg* fimbrial operon. We then used SCOTS in conjunction with microarrays to obtain the global expression profile of Typhi's genes in human macrophages compared to the supernatant of infection. This second approach has permitted the identification of 117 genes constitutively expressed during macrophage infection, of which 19 are specific to Typhi, including *clyA* and *STY1499*. This method also allowed the identification of most virulence factors already extrapolated from the murine model of typhoid fever, which are also used by Typhi during the infection of

human macrophages. We found that Typhi expresses the type three secretion system involved in macrophage survival and also many genes involved in antimicrobial peptide resistance. Next, we characterised and determined the roles of the *stg* fimbrial operon, *clyA* and *STY1499* in Typhi's pathogenesis. We found that Stg increases the adherence of Typhi to epithelial cells. However, it seems to reduce the phagocytosis of Typhi by human macrophages. ClyA seems to be cytotoxic for epithelial cells but not for macrophages. It also seems to reduce Typhi's survival in macrophages. This could be important to control the multiplication of Typhi and therefore allow the establishment of a systemic infection. STY1499 is a novel protein that is secreted by Typhi independently of both type three secretion systems. It seems to be involved in the invasion of phagocytic cells but not epithelial cells. STY1499 has therefore been renamed TaiA for 'Typhi associated invasin A'. In conclusion, the SCOTS technique, alone or in conjunction with microarrays, has identified specific Typhi genes involved in the interaction with human cells. The work presented in this thesis allows a better understanding of Typhi's pathogenesis and could help in the development of attenuated strains amenable to a use as vaccines or antigen delivery systems.

Key words: *Salmonella enterica* serovar Typhi, typhoid fever, systemic infection, host specificity, Selective Capture Of Transcribed Sequences (SCOTS), microarrays, virulence factors, fimbriae, hemolysin, invasin

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Å	Angstrom
aa	« Amino acid »
ADN	Acide désoxyribonucléique
ADNc	Acide désoxyribonucléique complémentaire
ADNg	Acide désoxyribonucléique génomique
ADNr	Acide désoxyribonucléique ribosomal
Ap	« Ampicillin »
ARN	Acide ribonucléique
ARNr	Acide ribonucléique ribosomal
ARNt	Acide ribonucléique de transfert
°C	Degré Celsius
Ca^{2+}	Calcium
CD	Cellules dendritiques
cDNA	« Complementary deoxyribonucleic acid »
CFTR	« Cystic transmembrane conductance regulator »
CFU	« Colony forming unit »
CGH	« Comparative genomic hybridization »
CO_2	« Carbon dioxide »
C_t	« Threshold cycle »
DAP	« DL-α,ε-diaminopimelic acid »
DOC	« Deoxycholic acid »
DMEM	« Dubelcco's modified Eagle's medium »
DNA	« Deoxyribonucleic acid »

EtOH	« Ethanol »
FCS	« Foetal Calf Serum »
FeII	Fer ferreux
FeIII	Fer ferrique
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
HA	« Hemmaglutinine »
HCL	« Hierarchical clustering »
HEPES	« *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid »
HGT	« Horizontal gene transfer »
IVET	« In vivo expression technology »
kb	Kilobases
kDa	Kilodalton
Kn	« Kanamycin »
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LiCl	« Lithium chloride »
LPM	« Low phosphate low magnesium medium »
LPS	Lipopolysaccharide
Mg^{2+}	Magnésium
$MgCl_2$	« Magnesium chloride »
Mn^{2+}	Manganèse
MEM	« Modified Eagle's medium »
min	Minute
ml	Millilitre

mM	Millimolaire
MOI	« Multiplicity of infection »
NaCl	Chlorure de sodium
OD	« Optical density »
OMV	« Outer membrane vesicle »
ORF	« Open reading frame »
PA	Peptides antimicrobiens
PBS	« Phosphate buffered saline »
PCR	Réaction en chaîne de la polymérase
PI	« Pathogenicity Island »
PMA	Phorbol 12-myristate 13-acetate
qPCR	« Quantitative real-time PCR »
RNA	« Ribonucleic acid »
RPMI	« Roswell Park Memorial Institute »
SCOTS	« Selective Capture Of Transcribes Sequences »
SCV	« *Salmonella* Containing Vacuole »
SPI	« *Salmonella* Pathogenicity Island »
SST3	Système de sécrétion de type 3
SST3-1	Système de sécrétion de type 3 codé sur le SPI-1
SST3-2	Système de sécrétion de type 3 codé sur le SPI-2
STM	« Signature-tagged mutagenesis »
TBST	« Tris buffered saline with tween »
TCS	« Two Component System » / système à deux composantes
T3SS/TTSS	« Type three secretion system »
U	« Unit »

À Dolorès

There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.

Charles Darwin
On the origin of species

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CHAPITRE 1 : Revue de littérature

1.1 Introduction

Les infections bactériennes sont une cause importante de morbidité et de mortalité tant dans les pays développés que ceux en développement. Les maladies causées par les bactéries sont multiples : pneumonies, gastro-entérites, septicémies et dermatites, pour ne nommer que celles-là. Depuis quelques années, les infections nosocomiales sont devenues un problème majeur. Un des meilleurs exemple est l'infection par *Clostridium difficile* et la peur qu'elle suscite chez les patients hospitalisés (42). De plus, les infections nosocomiales chez les nouveaux-nés sont une cause importante de mortalité même avec l'utilisation d'antibiotiques (135). Pour prévenir les infections bactériennes, les gouvernements ont établi des règles et des programmes de surveillance quant à la salubrité des aliments et de l'eau potable et de pratiques hospitalières.

Salmonella est un genre regroupant des espèces de bactéries pathogènes à Gram négatif intracellulaires facultatives qui sont utilisées depuis de nombreuses années comme modèles pour l'étude des mécanismes de pathogenèse. Les *Salmonella* causent chez les mammifères, y compris l'homme, des infections tant localisées que systémiques. Actuellement, plusieurs groupes de recherche tentent de mettre au point des vaccins pour lutter contre ces infections (90). De plus, plusieurs groupes essaient de développer une souche de *Salmonella* utilisable comme vecteur d'antigènes pour la

vaccination contre d'autres infections (5). Il est donc impératif de déterminer et de caractériser les mécanismes de pathogenèse de *Salmonella.*

1.2. *Salmonella*

Le genre *Salmonella* fait partie de la famille des *Enterobacteriaceae*, qui comprend les entérobactéries tel que le genre *Escherichia. Salmonella* partage environ 80 % de son génome avec son plus proche cousin *Escherichia* (55). De ce fait, la grande majorité des données actuelles concernant le métabolisme et le catabolisme de base ainsi que les mécanismes de transcription et de traduction d'*Escherichia coli* sont aussi valables pour *Salmonella.* De plus, les outils de biologie moléculaire développés pour étudier *E. coli* sont aussi applicables chez *Salmonella.*

1.2.1. *Salmonella bongori et Salmonella enterica*

Le genre *Salmonella* se divise en deux espèces, *Salmonella bongori* et *Salmonella enterica*. Du point de vue génomique, leur principale différence se situe au niveau de l'acquisition par *Salmonella enterica* d'un fragment génique codant pour un système de sécrétion de type trois (SST3) appelé SPI-2 (voir section 1.4.4) (162). L'espèce *enterica*, qui compte plus de 2000 sérovars, est la seule qui infecte les animaux à sang chaud (106, 172). Certains sérovars ont un spectre d'hôte large, d'autres sont restreints à un hôte particulier. Par exemple, le sérovar Typhimurium cause des infections chez une variété de mammifères, y compris l'homme, et chez la volaille, tandis que Typhi est restreint à l'homme. Seule une fraction des 2000 sérovars sont

fréquemment isolés chez l'homme. Par exemple, entre juin 2005 et mai 2006, les sérovars Typhimurium, Heidelberg, Enteritidis et Infantis représentaient les sérovars isolés dans 57 % des cas lors d'infections humaines au Canada (http://www.phac-aspc.gc.ca/c-enternet/index_f.html).

1.2.2. Pathologies associées à *Salmonella enterica*

Les salmonelloses sont souvent associées aux empoisonnements alimentaires et se transmettent par la voie fécale-orale. Ainsi, l'incidence des salmonelloses dans une société est inversement proportionnelle à son degré de salubrité alimentaire et de la qualité de l'eau potable. Au Canada, la majorité des salmonelloses humaines se présentent sous la forme d'une gastro-entérite et sont causées par des sérovars fréquemment retrouvés chez les animaux d'élevage et chez les animaux domestiques. Ces salmonelloses sont en fait des zoonoses. Au Canada, la source de contamination majeure est le poulet. Entre juin 2005 et mai 2006, seulement 156 cas de salmonelloses ont été rapportés (http://www.phac-aspc.gc.ca/c-enternet/index_f.html). Cependant, l'incidence réelle de salmonelloses au Canada est difficile à estimer, puisque les personnes infectées consultent rarement les instances médicales. *S. enterica* peut aussi causer des infections systémiques comme la fièvre typhoïde (115).

1.3. La fièvre typhoïde

La fièvre typhoïde est une infection systémique restreinte à l'homme (168). Le sérovar en cause, Typhi, a été décrit pour la première fois par Eberth en 1881 (11). Les

sérovars Paratyphi A, B et C causent une infection similaire, mais souvent moins sévère (18).

Le symptôme majeur accompagnant la fièvre typhoïde est une forte fièvre. D'autres symptômes peuvent aussi être présents : fortes céphalées, malaises, myalgie, nausée, anorexie et toux sèche (18). Les patients infectés peuvent aussi présenter des désordres intestinaux allant de la constipation à des diarrhées sanguinolentes dans les cas les plus graves (18). Le quart des patients infectés présentent aussi des lésions érythémateuses maculopapulaires. Chez les patients atteints de fièvre typhoïde, le diagnostic est souvent erroné puisque les symptômes sont très variables, peu spécifiques et qu'ils sont aussi présents dans des maladies comme la tuberculose, les septicémies et les hépatites (18).

1.3.1. Épidémiologie

Les plus récentes estimations font état d'environ 21 millions de cas de fièvre typhoïde et plus de 200 000 décès mondialement (43). La majorité des cas de fièvre typhoïde se retrouve dans les pays en développement où les mauvaises conditions sanitaires facilitent la transmission (115). L'homme est le seul réservoir de Typhi et la transmission se produit par l'ingestion de nourriture ou d'eau contaminée par les selles d'un individu infecté. L'incidence de la fièvre typhoïde est particulièrement élevée en Afrique subsaharienne (plus de 100 cas par 100 000 individus) et au sud de l'Asie dans les zones densément peuplées (18). Une incidence record de 980 cas par 100 000 individus a même été recensée à Delhi, en Inde (37). L'amélioration des conditions

d'hygiène dans les pays développés a permis d'enrayer les sources de transmission et Typhi n'est plus considérée comme endémique en Europe et en Amérique du Nord (37). Les principaux cas recensés dans les pays développés sont reliés au voyage dans les pays où la fièvre typhoïde est endémique (37)

Récemment, Typhi a été identifié comme la cause de la « peste » qui ravagea Athènes entre 436 et 420 av. J.-C., ce qui illustre l'impact que peut avoir la typhoïde sur la société humaine (166).

1.3.2. Pathogenèse

Une fois ingérée, Typhi traverse l'estomac et se retrouve dans le petit intestin où elle adhère à la muqueuse et envahit les cellules intestinales et les cellules M des plaques de Peyer (Fig. 1) (60, 168). Les bactéries sont alors relâchées dans les follicules lymphatiques intestinaux où elles sont phagocytées par les macrophages. À cette étape, Typhi se multiplie dans les macrophages de l'hôte, à l'intérieur d'un phagosome modifié appelé « *Salmonella* Containing Vacuole » (SCV). Typhi utilise le macrophage pour se disperser dans l'organisme et infecter une variété d'organes tels le foie, la rate et la moelle osseuse (18). Après 7 à 14 jours d'incubation, les bactéries sont relâchées dans la circulation sanguine. Il s'agit alors de la phase de bactériémie pendant laquelle les symptômes apparaissent : fièvre, fortes céphalées, nausée et douleur abdominale (168).

L'emplacement exact des bactéries dans les organes infectés est encore matière à débat, mais certaines études suggèrent que les bactéries sont majoritairement retrouvées

à l'intérieur des macrophages résidents et qu'ils sont en fait le réservoir de Typhi (182, 187). La charge bactérienne dans la moelle osseuse a un effet déterminant sur l'issue de l'infection puisque les complications sont plus fréquentes lorsque la charge est élevée (216).

Suite à l'infection du foie, les bactéries infectent la vésicule biliaire, ce qui leur permet d'être sécrétées avec la bile dans le petit intestin (Fig. 1). Ces bactéries sont excrétées avec les matières fécales, ce qui permet la transmission à un autre individu. Les bactéries sécrétées par la vésicule biliaire peuvent aussi envahir de nouveau le petit intestin. Cette seconde vague d'invasion déclenche, dans les cas graves, une réponse immunologique excessive qui peut entraîner la perforation de l'épithélium intestinal et la mort (60, 168). Dans 2 à 5 % des cas, les personnes infectées deviennent porteurs chroniques, même si elles ont été traitées (37). Un lien causal a été établi entre le cancer de la vésicule biliaire et l'infection chronique par Typhi (179). Les porteurs chroniques sont d'une importance capitale pour la transmission de la maladie puisqu'ils servent de réservoir. Ils ne présentent pas de symptômes et, faute de traitement, peuvent demeurer porteurs à vie (37). Au début du 20^{e} siècle, des détectives en épidémiologie ont été formés pour combattre les épidémies de fièvre typhoïde en identifiant les porteurs chroniques (115). L'un de ces détectives, Georg Soper, a été embauché par la ville de New York pour investiguer une épidémie de fièvre typhoïde qui s'est déclarée dans la résidence d'une famille fortunée de Long Island. La cuisinière, Mary Mallon, a été identifiée comme la cause de l'épidémie, puisque des cas de fièvre typhoïde ont été recensés dans plusieurs demeures où Mary Mallon travaillait. Il était donc clair que celle-ci était en fait la porteuse chronique qui permettait la transmission de la typhoïde.

Après son refus de se soumettre au test diagnostic, elle fut incarcérée et obligée de se soumettre aux tests qui se révélèrent positifs. Elle fut relâchée trois ans plus tard sous la condition de ne plus exercer le métier de cuisinière. Georg Soper découvrit cinq ans plus tard qu'elle avait recommencé ce métier et elle fut donc incarcérée de nouveau jusqu'à sa mort en 1938.

1.3.3. Prévention et traitement

Historiquement, la fièvre typhoïde a été traitée avec l'ampicilline et le chloramphénicol. Toutefois, en 1989, presque toutes les souches isolées de Typhi étaient résistantes à ces antibiotiques et leur utilisation a été abandonnée (37). Les fluoroquinolones (ciprofloxacine et olfloxacine) ont été ensuite les antibiotiques de choix pour traiter la typhoïde (168). Cependant, plusieurs souches de Typhi isolées dernièrement en Asie sont résistantes à ces antibiotiques (12). La gatifloxacine, un fluoroquinolone nouveau genre, est actuellement suggéré dans les cas de résistance. Cet antibiotique est particulièrement utile dans les pays en développement puisqu'il est relativement peu coûteux (12). Dernièrement, la susceptibilité à de vieux antibiotiques tels que le chloramphénicol semble être réapparue et il peut être utilisé dans certaines régions, particulièrement en Asie où le chloramphénicol est très peu coûteux (12). De façon générale, l'antibiothérapie utilisée pour traiter la fièvre typhoïde est en constant changement, ce qui reflète la capacité des bactéries pathogènes à s'adapter aux antibiotiques utilisés.

La prévention de la fièvre typhoïde passe en premier lieu par l'amélioration des conditions sanitaires et l'accès à l'eau potable, ce qui est un problème dans les zones endémiques où les infrastructures sont peu développées. Puisque la fièvre typhoïde est restreinte à l'homme et surtout parce que l'homme constitue le seul réservoir, cette maladie pourrait être éradiquée, à condition de disposer d'un vaccin efficace, comme pour la poliomyélite (145, 195). Deux types de vaccins sont actuellement utilisés chez les personnes qui voyagent dans des zones endémiques. Le premier est constitué de la souche Ty21a et est administré par la voie orale (18). Ty21a est une souche atténuée préparée en traitant la souche Ty2 avec de la nitrotrosoguanidine qui est un puissant générateur de mutation. La souche Ty21a est donc mal définie génétiquement. Le second est composé de l'antigène Vi, la capsule polysaccharidique de Typhi (voir section 1.4.6.1), qui est administré par voie intramusculaire (18). En général, les deux vaccins sont efficaces dans une proportion d'environ 75 % sur une période maximale de 3 ans. Malheureusement, l'immunité fournie par ces vaccins peut être contournée par une forte dose d'infection, qui est courante dans les zones endémiques. Pour ces raisons, plusieurs groupes cherchent à mettre au point un vaccin plus efficace (75, 90). L'un d'eux est composé de la souche Ty800 qui contient une mutation du TCS PhoP/PhoQ (voir section 1.5.1.1). Cette souche semble être immunogénique et bien tolérée, mais son efficacité reste à démontrer (75, 104). Cette souche a aussi été testée comme vecteur d'antigène pour l'immunisation contre l'uréase de *Helicobacter pylori*. Malheureusement, aucun des volontaires n'a développé d'immunité contre l'uréase et il semble que la souche Ty800 soit trop atténuée pour permettre une immunisation efficace (51). Bien que plusieurs autres souches atténuées présentent une bonne efficacité immunogénique, leurs

efficacités sur le terrain restent à démontrer. Il est donc important de développer constamment de nouvelles souches pour avoir des solutions de réserve (75).

L'identification de cibles thérapeutiques pourrait ouvrir la voie à l'élaboration d'antibiotiques utilisant de nouveaux modes d'action ou d'antibiotiques spécifiques pour les bactéries pathogènes (22). Il a été suggéré que les facteurs de virulence sont des cibles thérapeutiques envisageables (2). Bien que les antibiotiques dirigés contre les facteurs de virulence ne sont pas bactéricides, l'inhibition de facteur de virulence important devrait aider le système immunitaire de l'hôte à se débarrasser de l'agent infectieux. De plus, de tels antibiotiques sont moins susceptibles d'affecter la flore normale de l'hôte. Dernièrement, des dérivés d'une molécule appelée salicylidene acylhydrazide se sont révélés efficaces pour inhiber les SST3 de *Salmonella* (161). Ces inhibiteurs réduisaient les phénotypes d'invasion et de survie intracellulaire qui sont dépendants des SST3 et empêchaient la sécrétion d'effecteur par ces systèmes (voir section 1.4.3. et 1.4.4.) (161). De plus, ces inhibiteurs se sont révélés efficaces pour prévenir le développement de l'infection de souris par Typhimurium (110).

L'identification de cibles potentielles dépend de la connaissance des mécanismes de pathogenèse. La prévention des infections par l'utilisation de vaccins est elle aussi une approche efficace pour lutter contre les infections bactériennes, mais leur développement dépend aussi de la connaissance des mécanismes de pathogenèse. De ce fait, l'identification des facteurs de virulence et des systèmes métaboliques propres à une bactérie pathogène donnée, lors de l'infection, est cruciale pour le développement d'antibiotiques spécifiques et de vaccins efficaces.

1.4. Facteurs de virulence de Typhi

Puisque Typhi est restreint à l'homme, il n'existe pas de modèle animal pour l'étudier. Par contre, Typhimurium cause une infection systémique mortelle chez certaines lignées de souris. Les lignées de souris utilisée dans le modèle murin de la fièvre typhoïde sont habituellement des BalB/c qui ont une mutation dans le gène Nramp1. L'infection de souris *Nramp*$^{+/+}$, comme les 129sv, résulte en une infection généralement asymptomatique où les souris peuvent être porteuses pour plusieurs mois (152). Les chercheurs ont donc utilisé le modèle murin pour trouver les facteurs de virulence utilisés par *Salmonella* lors de l'infection systémique. Ainsi, la grande majorité des facteurs de virulence connus de Typhi ont été extrapolés des recherches effectuées chez Typhimurium. Par contre, la prudence est de mise puisque certains facteurs de virulence requis par Typhimurium pour l'infection systémique de la souris sont absents ou présents sous la forme de pseudogènes chez Typhi, comme l'opéron *spv* qui est situé sur le plasmide de Typhimurium (85).

Les lignées de cellules en culture se révèlent un bon modèle pour étudier directement Typhi. Par exemple, l'utilisation des lignées humaines de monocytes comme les THP-1 ou les U937 permettent d'identifier des facteurs nécessaires à la survie et à la multiplication de Typhi dans les macrophages. Il est généralement accepté que les macrophages constituent le réservoir de Typhi pendant l'infection (182).

1.4.1. « *Salmonella* Pathogenicity Island » (SPI)

Les îlots de pathogenicité (PI) sont des régions génomiques, présentes chez plusieurs bactéries pathogènes qui contiennent des facteurs de virulence (97). Chez une même espèce, ces PI sont présents chez les souches pathogènes, mais sont habituellement absents chez les souches non pathogènes. Les PI ont généralement un contenu en GC différent du reste du génome et sont parfois associés à des sites d'insertion comme des ARNt. Dans certains cas, des fonctions de mobilités de l'ADN peuvent être codées sur les PI, comme des gènes de bactériophages, des intégrases et des transposases (192). Ainsi, les PI permettent l'acquisition « en bloc » de facteurs de virulence provenant d'autres espèces par transfert horizontal de gènes et permettent donc des bonds évolutifs (83).

Chez *Salmonella*, les PI sont appelés SPI pour « *Salmonella* Pathogenicity Island ». Le génome de Typhi en compte 10 (167). SPI-1 et SPI-2 codent pour les protéines nécessaires à des systèmes de sécrétions de type trois (SST3) qui sont impliqués respectivement dans l'invasion et la survie dans les cellules de l'hôte (voir section 1.4.2 à 1.4.4). Les autres SPI de Typhi qui contiennent des facteurs de virulence connus seront discutés plus loin.

1.4.2. Système de sécrétion de type trois (SST3)

Le SST3 est une structure en aiguille (Fig. 2) qui est utilisée par de nombreuses bactéries pathogènes pour injecter des protéines directement à l'intérieur de l'hôte (77,

119). Bien que cette structure s'apparente à la structure des flagelles, ces deux systèmes ont probablement évolué de façon indépendante à partir d'un ancêtre commun (79). Les SST3 sont strictement retrouvés chez les bactéries à Gram négatif (77). Par exemple, plusieurs bactéries infectant les animaux possèdent un SST3 comme *E. coli, Shigella, Chlamydia, Vibrio* et *Pseudomonas*. Certaines en possèdent même deux, comme *Salmonella* et *Yersinia*. Le SST3 est aussi présent chez les bactéries pathogènes de végétaux, comme *Erwinia* et certaines espèces de *Pseudomonas*. Chez toutes ces bactéries pathogènes, le SST3 est un facteur de virulence majeur.

1.4.3. Le SST3 codé sur le SPI-1 et l'invasion des cellules

La première caractéristique majeure du genre *Salmonella* est la capacité à envahir les cellules non phagocytaires de l'hôte. Cette faculté leur permet de pénétrer les cellules épithéliales composant la barrière intestinale de l'hôte pour établir une infection des tissus intestinaux (dans le cas de gastro-entérite) ou une infection systémique (dans le cas de la fièvre typhoïde). En général, le SPI-1 est considéré comme le déterminant majeur de l'invasion puisqu'il code le SST3 (SST3-1) requis et certains des effecteurs qu'il transporte (67). Galàn et Curtiss (69) ont, les premiers, identifié certains des gènes (*invABCD*) impliqués dans le phénotype d'invasion en utilisant une approche de complémentation d'un mutant défectif pour l'invasion. Ils ont aussi démontré qu'une souche Δ*invA* de Typhimurium avait, dans un modèle murin d'infection, une dose létale plus élevée par inoculation par voie orale, mais une dose létale inchangée par voie intraveineuse. *invA*, et par conséquent le SST3-1, est donc impliqué dans la phase

intestinale de l'infection, pour traverser la barrière intestinale. Par contre, il n'est pas impliqué dans la phase systémique de l'infection.

SPI-1 est constitué d'environ 40 kb d'ADN insérés entre les gènes *mutS* et *fhlA* qui sont consécutifs chez *E. coli* K-12 (150). Il code aussi pour le transporteur de manganèse *sitABCD* qui est impliqué dans la virulence de Typhimurium chez la souris (112, 116). Le SST3-1 est composé de 35 gènes qui codent pour des protéines structurales, des chaperonnes, des effecteurs et des régulateurs. La régulation du SST3-1 est complexe (Fig. 3) et requiert 5 protéines. La plus importante est HilA puisque son promoteur agit comme un intégrateur de signaux (134). Lorsque les conditions sont favorables, HilD, HilC et RtsA permettent la transcription de *hilA* (57). HilA induit l'expression des opérons *prg*, *inv* et *spa*, qui codent en grande partie pour des protéines structurales du SST3-1 (134). InvF se lie ensuite à SicA, ce qui permet l'expression des effecteurs transportés par le SST3-1 dont l'opéron *sip* (46, 47).

Les conditions qui activent le SST3-1 sont nombreuses. Son expression nécessite une osmolarité élevée et une faible aération, qui sont probablement les conditions rencontrées dans le petit intestin (7, 68). La présence de bile semble aussi pouvoir induire l'expression de SST3-1, via le système à deux composantes (TCS) BarA/SirA (174).

À ce jour, au moins 14 protéines sont reconnues pour être transportées par le SST3-1. AvrA, SipA, SipB, SipC, SipD et SptP sont codées dans le SPI-1, SopB dans le SPI-5, SopE dans le SPI-7 et SlrP, SopA, SopC, SopD, SopE2 et SspH1 sont distribuées

dans le génome, mais ne se retrouvent pas dans les SPIs (139). Ceci illustre que les SST3 sont capables de transporter des produits de gènes qui ont été acquis indépendamment. Le mécanisme de reconnaissance des effecteurs transportés par les SST3 est encore mal défini (70). Dans certain cas, il semble y avoir un signal de sécrétion dans les premiers 20 à 30 acides aminés. Par contre, ce signal n'est pas conservé chez tous les effecteurs transportés par les SST3 et il n'y a pas de signal consensus. De plus, il semble que certains effecteurs n'ont pas de signal au niveau de la séquence d'acide aminé ce qui a mené à l'élaboration d'une hypothèse suggérant que le signal se retrouverait au niveau de l'ARN messager (4). De plus, il semble que des chaperonnes soient impliquées dans la reconnaissance de certains effecteurs (128, 218). La reconnaissance des effecteurs est donc très complexe et pourrait impliquer plusieurs mécanismes.

L'invasion de cellules non phagocytaires est strictement dépendante du réarrangement de l'actine et plusieurs effecteurs transportés par le SST3-1 influencent la polymérisation de l'actine (139). Par exemple, SopE et SopE2 sont des facteurs d'échange de guanine qui agissent spécifiquement sur les GTPases Rac et Cdc42 (94, 200). Les GTPases sont des interrupteurs moléculaires qui sont actifs lorsque liés au GTP, mais inactifs lorsqu'ils ont hydrolysé le GTP en GDP. Les facteurs d'échange de guanine permettent de remettre l'interrupteur à son état activé en échangeant le GDP pour un GTP. Puisque Rac et Cdc42 régulent le réseau d'actine, SopE et SopE2 permettent en fait à *Salmonella* de contrôler indirectement ce réseau.

Outre l'invasion des cellules non phagocytaires, le SST3-1 induit la sécrétion de fluide et l'infiltration de neutrophiles dans la lumière intestinale. Ces fonctions ont été étudiées chez Typhimurium dans le modèle bovin de gastro-entérites et ont été attribuées à six effecteurs : SipA, SopA, SopB, SopD, SopE et SopE2 (226, 227). Il serait tentant d'extrapoler ces découvertes à Typhi. Il faut cependant tenir compte du fait que Typhi ne cause pas de gastro-entérite et que la diarrhée n'est pas un symptôme reconnu de la fièvre typhoïde (37). En fait, chez Typhi et Paratyphi, SopA, ainsi que certains gènes impliqués dans la colonisation intestinale par Typhimurium, sont des pseudogènes (145). De plus, SipA, SopB et SopD de Typhi présentent certaines mutations par rapport à leurs homologues chez Typhimurium. Cependant, les effecteurs SipA, SopB et SopD de Typhi sont fonctionnels et peuvent complémenter un mutant *sipA sopABDE2* de Typhimurium (178). La différence entre Typhi et Typhimurium au niveau de l'induction de sécrétion de fluide dans la lumière intestinale devra donc être expliquée d'une autre façon. L'implication de la capsule Vi de Typhi à ce niveau sera discutée plus loin (voir section 1.4.6.1).

Le SST3-1 est aussi impliqué dans la mort rapide des macrophages de l'hôte. Cet effet est associé à SipB qui se lie à la caspase-1 et induit l'apoptose précoce des macrophages (31, 100, 138). L'apoptose des macrophages dépendante de SPI-1 a récemment été appelée pyroptose, puisqu'elle est accompagnée de la libération de cytokines pro-inflammatoires, contrairement à l'apoptose (64).

1.4.4. Le SST3 codé sur le SPI-2 et la survie dans les macrophages

La deuxième caractéristique majeure de *S. enterica* est sa capacité à survivre à l'intérieur des macrophages de l'hôte. C'est en cherchant des gènes impliqués dans cette fonction que Ochman *et coll.* (163) ont identifié 2 gènes, *spiAB* (*ssaCD*), qui ressemblait à des gènes codant pour des protéines structurales de SST3. Il venait d'identifier le deuxième SST3 (SST3-2) codé sur le SPI-2 (Fig. 2). Fait intéressant, une souche Δ*spiA* (*ssaC*) survit moins bien dans les macrophages que la souche sauvage et est avirulente dans le modèle murin, qu'elle soit inoculée par voie orale ou par voie intraveineuse (163). De plus, des mutants du SST3-2 sont encore capables de coloniser la muqueuse intestinale, mais sont incapables de coloniser les ganglions lymphatiques, le foie et la rate (32). Cet effet est radicalement différent de l'effet d'une mutation du SST3-1 et démontre que le SST3-2 est important lors de la phase systémique de l'infection. Par contre, une étude récente a montré que le SST3-2 était aussi important pour l'établissement de l'infection intestinale dans un modèle murin de gastro-entérite pour lequel les souris sont traitées à la streptomycine (34). Ceci suggère que le SST3-2 pourrait aussi être impliqué dans la phase intestinale de l'infection.

Le SPI-2 a une taille d'environ 40 kb divisible en deux parties. Une première région de 25 kb code pour le SST3-2 et a un contenu en GC d'environ 43%. Elle est retrouvée seulement chez *S. enterica*. Une seconde région, d'environ 15 kb, possède un contenu en GC de 54 % et est présente chez *S. enterica* et *S. bongori*. Cette région code un système de réductase de tétrathionate (*ttr*) qui ne semble pas être impliqué dans la virulence (30). L'ajout du SST3-2 à *S. bongori* augmente sa survie à l'intérieur de

macrophages par rapport à la souche sauvage, mais ne permet pas de causer une infection systémique dans la souris (92). D'autres facteurs de virulence sont donc impliqués dans l'infection systémique.

L'expression du SST3-2 est régulée par SsrA/SsrB, codé dans le SPI-2. La première preuve est qu'une souche Δ*ssrA*(*spiR*) produit beaucoup moins SpiA (SsaC), une protéine structurale du SST3-2, que la souche sauvage (163). De plus, une souche Δ*ssrA* ou Δ*ssrB* est incapable d'induire l'expression de *sseA*, qui code pour une protéine sécrétée (99). L'expression du SST3-2 est induite *in vitro* dans des conditions de pH légèrement acide et en concentration faible de magnésium et de phosphate (16, 39, 49). L'expression de *ssrA/ssrB* est influencée par OmpR/EnvZ et PhoP/PhoQ (voir section 1.5.1.1 et 1.5.1.3 et Fig. 3) (49, 74).

Plusieurs fonctions ont été attribuées aux effecteurs transportés par le SST3-2. L'inhibition de la fusion de lysosomes avec la SCV a été attribuée à SpiC (194, 210). Or, la présence de SpiC dans les cellules de l'hôte n'a pas été détectée et il a été suggéré que cette protéine contrôlerait la sécrétion des effecteurs par le SST3-2 (224). Le rôle de SpiC dans l'inhibition de la fusion de lysosomes avec la SCV est donc matière à débat, mais l'effet global observé peut quand même être attribué au SST3-2. Deux autres effecteurs, SseF et SseG, semblent être requis pour que la SCV soit correctement positionnée près du Golgi (50). De plus, une souche Δ*sseF* ou Δ*sseG* est moins apte à se répliquer dans les cellules de l'hôte. SifA est impliquée dans le maintien de l'intégrité de la SCV puisqu'une souche Δ*sifA* est relâchée dans le cytoplasme de la cellule hôte et est incapable de survivre dans les macrophages (17, 26). Par contre, la protection conférée

par SifA est limitée, car une faible proportion de *Salmonella* sauvage se retrouve dans le cytoplasme où elles se couvrent d'ubiquitine et sont dirigées vers le protéasome pour dégradation (169).

Le SST3-2 semble aussi être impliqué dans l'inhibition de la réponse immunitaire. Tout d'abord, le SST3-2 bloque la présentation antigénique par les cellules dendritiques (CD) (206). Cet effet a été démontré en utilisant un système de présentation de l'ovalbumine. Des CD ont été infectées par la souche sauvage et des mutants du SST3-2 (Δ*spiA* et Δ*spiC*) exprimant l'ovalbumine. Seules les CD infectées par les mutants présentaient l'ovalbumine aux lymphocytes T spécifiques de l'ovalbumine. De plus, le SST3-2 semble pouvoir supprimer la production de cytokines par les cellules infectées en induisant l'expression de SOCS-3 (211). SOCS-3 bloque la production de cytokines en bloquant l'activité de la voie de signalisation JAK/STAT (199).

Dernièrement, le SST3-2 a été impliqué dans la progression de l'infection au niveau systémique. En effet, il a été démontré que l'effecteur SrfH augmentait la migration des macrophages infectés vers la rate et le foie. Cet effet est causé par l'interaction de SrfH avec TRIP6, qui module l'activité de la voie de signalisation Rac impliquée dans la motilité (221).

Le SST3-2 est impliqué dans la cytotoxicité envers les cellules de l'hôte. Contrairement à la cytotoxicité induite par le SST3-1, celle induite par le SST3-2 est plus tardive (212). Cet effet a récemment été attribué, du moins en partie, à SseL, qui possède une activité déubiquitinase (185). Le lien entre cette activité et la cytotoxicité

n'est pas encore établi, mais elle semble être importante pour l'infection systémique dans le modèle murin de la fièvre typhoïde (40).

1.4.5. SPI-3 et le transport du magnésium *(mgtBC)*

Le SPI-3 est un segment d'environ 17 kb inséré au site de l'ARNt *selC* (23). Deux systèmes de virulence connus sont codés sur le SPI-3. Premièrement, les gènes *mgtBC* sont requis pour la multiplication intracellulaire et l'établissement d'une infection systémique (23). L'expression de *mgtBC* est dépendante de PhoP/PhoQ qui semble détecter la concentration en magnésium à l'extérieur de la bactérie (voir section 1.5.1.1) (24). *mgtB* code pour un système de transport de magnésium de haute affinité qui est une ATPase de type P (196). Le transport de magnésium a aussi été attribué à *mgtC* du fait qu'il est requis pour que la bactérie puisse se multiplier dans des conditions où la concentration de magnésium est basse (23). Par contre, il a été démontré, en utilisant le système d'oocytes de *Xenopus laevis*, que *mgtC* ne transporte pas directement le magnésium, mais qu'il active les ATPase endogène de l'hôte, ce qui semble être avantageux pour survivre dans les macrophages (1, 87).

Le deuxième facteur de virulence connu codé sur le SPI-3 est MisL, une protéine de type autotransporteur (24). L'inactivation de *misL* par un transposon ne compromet pas la capacité de causer une infection systémique et n'est pas requise pour la survie dans les macrophages (24). Une étude récente a par ailleurs démontré que MisL est impliquée dans la colonisation intestinale des souris (53). Cet effet semble être dû à la capacité de MisL de se lier à la fibronectine, ce qui entraîne une augmentation de

l'invasion des cellules intestinales (53). L'expression de MisL est régulée par MarT qui est aussi codée dans le SPI-3 (209).

1.4.6. SPI-7

Le SPI-7 est spécifique aux sérovars Typhi, Paratyphi C et Dublin (172). Il est inséré à côté de l'ARNt *pheU* et est le plus grand de tous les SPI avec ses 134 kb (97). Le SPI-7 présente une structure particulièrement complexe et a probablement été construit par l'insertion indépendante d'éléments acquis par transfert horizontal de gènes (171). Par exemple, il contient le phage SopE (SopEΦ) de la famille du bactériophage P2, qui code l'effecteur SopE du SST3-1 (151). Bien que le SPI-7 soit absent du génome de Typhimurium, elle possède quand même en partie SopEΦ (204). Il a été suggéré que le SPI-7 provenait en partie d'un plasmide ou d'un transposon conjugatif (171). Par conséquent, le SPI-7 est instable et cet îlot est absent ou partiellement retrouvé chez certains isolats cliniques de Typhi (28, 159). Outre SopE, le SPI-7 code pour deux facteurs de virulence connus, soit la capsule de polysaccharide Vi et le pili de type IVB.

1.4.6.1. La capsule Vi

La capsule de polysaccharide Vi est un polymère d'acide galacturonique, avec un groupement *N*-acétyle à la position C-2 et un groupement acétyle variable à la position C-3, relié par des liaisons α(1→4) (Fig. 4) (201). La production et l'exportation de la capsule Vi dépendent d'un groupe de gènes appelé l'opéron *viaB*. Il contient les gènes *tviBCDE* qui codent pour les protéines nécessaires à la synthèse de la capsule Vi (215).

Le transport vers l'extérieur est assuré par un transporteur de type ABC codé par les gènes *vexABCDE* contenus dans l'opéron *viaB* (95). La production de la capsule Vi est contrôlée par TviA (215). L'expression de *tviA* est contrôlée par RcsB/RcsC et OmpR/EnvZ qui répond à l'osmolarité du milieu (voir section 1.5.1.3 et Fig. 3) (170, 214). Récemment, le facteur sigma RpoS a été identifié comme un autre régulateur de l'opéron *viaB* et est nécessaire pour une régulation adéquate de l'expression de Vi par rapport à l'osmolarité (188).

L'importance reconnue de la capsule Vi dans la pathogenèse de Typhi vient du fait que les souches Vi^- sont moins virulentes que les souches Vi^+ lorsqu'inoculées à des volontaires humains (107). De plus, une souche Δ*viaB* survit moins bien que la souche sauvage dans les macrophages humains en culture (102). Fait intéressant, les macrophages infectés par la souche sauvage produisent beaucoup moins de TNF-α que ceux infectés par la souche mutante (102). Le TNF-α est une cytokine pro-inflammatoire produite surtout par les phagocytes et permet le recrutement des neutrophiles et des phagocytes au site d'infection (122). Ainsi, la répression de la production de TNF-α constitue probablement un moyen de réduire la réponse immunitaire de l'hôte. La capsule Vi semble aussi diminuer la production de la cytokine pro-inflammatoire IL-8 par les cellules intestinales et les macrophages (175). Récemment, il a été démontré qu'une souche de Typhimurium exprimant la capsule Vi réduisait la réponse inflammatoire et la production de la cytokine pro-inflammatoire IL-17 par l'épithélium gastrique dans le modèle de boucle iléale ligaturée de bovin (177). Ainsi, la capsule Vi permet à Typhi de réduire la réponse inflammatoire au niveau de l'intestin, ce qui expliquerait la faible propension de Typhi à causer des diarrhées (176).

1.4.6.2. Le pili de type IVB

Le pili de type IVB de Typhi fait partie des facteurs de virulence qui permettent l'adhésion aux cellules de l'hôte. Bien que ce sujet sera abordé plus en détail à la section 1.4.10, le pili de type IVB sera discuté ici puisqu'il est codé sur le SPI-7 et qu'il est régulé comme la capsule Vi (127).

Le pili de type IVB est impliqué dans l'invasion des cellules intestinales et des macrophages (165, 228). Il a été démontré que ce pili aide l'adhérence aux cellules intestinales en se liant au récepteur « cystic fibrosis transmembrane conductance regulator » (CFTR), ce qui permet par la suite au SST3-1 d'induire l'invasion (208). Typhi semble être capable de provoquer la distribution de CFTR à la surface des cellules intestinales, ce qui favorise la liaison par le pili de type IVB (140). Par contre, le facteur bactérien impliqué dans ce phénomène n'a pas été identifié.

1.4.7. Les lipopolysaccharides

La surface externe des bactéries Gram négatif, comme *Salmonella*, est composée de lipopolysaccharides (LPS) qui sont formés de trois sous-unités. Le lipide A, hydrophobe, qui est à la base du LPS, sert de point d'attache dans la membrane externe (52). Vient ensuite, la région centrale composée d'oligosaccharides phosphorylés, puis l'antigène O, composé d'une suite d'oligosaccharides qui se dressent à la surface de la bactérie (52). La longueur de l'antigène O est régulée chez *Salmonella* par deux

protéines, Wzz et FepE (158). Il semble que la longueur de la chaîne O influence la phagocytose par les macrophages et l'activation du complément (157). Une longueur entre 16 et 35 sous-unités permet une phagocytose maximale et une activation minimale du complément. De plus, la survie dans les macrophages est favorisée par les LPS courts (21). Par contre, l'entrée dans les cellules intestinales semble être influencée par les oligosaccharides de la région centrale plutôt que par l'antigène O (103).

En réponse à la présence de lipide A, les cellules de l'hôte produisent certaines cytokines pro-inflammatoires et induisent l'expression de certaines adhésines qui sont impliquées dans plusieurs mécanismes immunitaires (59). Pendant les infections bactériennes, l'exposition des cellules de l'hôte au lipide A est responsable de l'apparition de symptôme telle que la fièvre (59).

1.4.8. Résistance aux peptides antimicrobiens

Les peptides antimicrobiens (PA) sont des chaînes polypeptidiques de moins de 100 acides aminés sécrétées par diverses cellules (71). Les PA sécrétés par les mammifères peuvent être classés en deux catégories : les défensines et les cathélicidines. Toutes deux sont produites par les cellules épithéliales et les phagocytes et sont retrouvés à des concentrations de l'ordre du millimolaire dans les tissus (72, 225). Les PA tuent les bactéries à Gram négatif en perméabilisant séquentiellement la membrane externe puis la membrane interne (129). Pour se faire, les PA, chargés positivement, sont d'abord attirés par les charges négatives présentent à la surface de la bactérie. Les PA s'insèrent ensuite dans la membrane. À ce sujet, plusieurs modèles ont été proposés,

dont ceux du tore, du baril et du tapis (Fig. 5) (25). Ces trois modèles sont maintenant perçus comme étant des étapes successives de l'insertion des PA dans la membrane (48).

Il est impératif pour les bactéries pathogènes de résister aux PA produits par l'hôte. En ce sens, *Salmonella* est capable de détecter la présence de PA et d'initier les mécanismes de résistance appropriés. La détection se fait via le système à deux composantes PhoP-PhoQ qui induit l'expression des gènes nécessaires (voir section 1.5.1.1 et Fig. 3). Plusieurs protéines impliquées dans la résistance aux PA modifient les LPS pour en diminuer la charge négative. PmrE (Ugd) et PmrF catalysent l'ajout d'un groupement 4-aminoarabinose ou phosphoéthanolamine au phosphate du lipide A (229) et PagP ajoute un groupement palmitate au lipide A (89). La résistance aux PA est aussi accomplie par leur dégradation. PgtE agit en ce sens en clivant les PA de la famille des cathélicidines (84).

Les modifications des LPS décrites ci-dessus semblent aussi être impliquées dans un changement de la réponse immunitaire de l'hôte. Il a été démontré que les LPS purifiés d'une souche de Typhimurium qui possédait un allèle de PhoP constitutivement active ($PhoP^c$) étaient moins inflammatoires que les LPS isolés d'une souche sauvage (88). Les LPS isolés de la souche $PhoP^c$ présentent des modifications par rapport aux LPS de la souche sauvage comme l'addition de 4-aminoarabinose. De plus, il a été démontré que les LPS de *Salmonella* isolées de bactéries récoltées tout juste après l'infection de macrophages présentaient aussi de telles modifications (78). Ces observations viennent confirmer l'importance des modifications des LPS pour la pathogenèse de *Salmonella*.

1.4.9. Transport du fer

Le fer est un élément indispensable aux bactéries et il est généralement reconnu que les systèmes de transport du fer sont des systèmes de virulence importants (35). Des études épidémiologiques ont montré que les patients souffrant de surcharge en fer, comme la thalassémie, sont plus susceptibles aux infections bactériennes (217). Dans des conditions anaérobiques à pH 7, le fer est sous sa forme réduite soluble (FeII) et diffuse librement à travers les membranes. Chez *Salmonella* et *E. coli*, le FeII peut être transporté par le transporteur à haute affinité codé par les gènes *feoABC* (113). Par contre, à pH 7 dans des conditions aérobiques, le fer est sous sa forme oxydée insoluble (FeIII). De plus, le fer n'est pas libre dans l'hôte et est lié à des protéines comme la ferritine et la transferrine (180). Le fer est donc un élément difficile à acquérir pour les bactéries pathogènes et elles utilisent à cette fin plusieurs mécanismes, y compris des systèmes de transport complexes appelés sidérophores. Les bactéries entériques comme *Salmonella* utilisent l'entérobactine qui est synthétisée par les protéines codées par *entA-F*. Le système de perméase qui permet l'acquisition du complexe lié au fer est codé par les gènes *fepA-G* (180). La salmochélline utilisée par *Salmonella* est produite par la glycosylation de l'enterobactine (20). Sa synthèse et son transport sont dépendants des gènes *iroBCDEN* codés sur le SPI-9 (14, 93). La transcription des gènes impliqués dans le transport du fer est régulée par la protéine Fur. Lorsque le fer est disponible à l'intérieur de la bactérie, Fur se lie au FeII et réprime la transcription des gènes impliqués dans la synthèse et dans le transport des sidérophores (180).

1.4.10. Adhésion aux cellules de l'hôte

L'attachement aux cellules de l'hôte et aux muqueuses est une étape essentielle de la colonisation de l'hôte. Les fimbriae, pili et adhésines sont les facteurs de virulence qui permettent l'attachement des bactéries aux cellules de l'hôte, aux muqueuses et aux surfaces abiotiques. Par exemple, chez Typhi, MisL et le pili de type IVB, discutés aux sections 1.4.5 et 1.4.6.2, sont impliqués dans l'adhésion aux cellules de l'hôte.

Typhi possède 12 opérons fimbriaires : *bcf, csg, fim, saf, sef, sta, stb, stc, std, ste, stg, sth* et *tcf* (207). Par contre, les fimbriae *bcf, fim, saf, sef, stb, ste, stg* et *sth* contiennent des pseudogènes et sont probablement non fonctionnels (145). Par immunobuvardage de Southern, il a été démontré qu'aucun de ces fimbriae n'est spécifique à Typhi ou aux autres sérovars qui causent la fièvre typhoïde (207). Par contre, une seule sonde a été utilisée pour chaque fimbriae. Ainsi, un fimbriae présent dans deux sérovars peut avoir une spécificité de liaison différente si la séquence de l'adhésine est différente, grâce à une mutation ponctuelle par exemple. De telles mutations, appelées « Single Nucleotide Polymorphism », sont sélectionnées si elle confère un avantage évolutif (220). Par exemple, le fimbriae *fim* est présent chez tous les sérovars. Chez Typhimurium, il code un fimbriae de type 1 qui permet de lier l'hémagglutinine via le mannose, alors que chez Gallinarum il code pour un fimbriae de type 2 qui est incapable de lier le mannose (207). Chez Typhi, *fimI* est un pseudogène et le fimbriae *fim* est probablement non fonctionnel (145).

Les fimbriae de Typhi sont très peu caractérisés et, comme pour les autres facteurs de virulence de Typhi, les données disponibles ont été obtenues en étudiant Typhimurium ou d'autres sérovars apparentés. Chez Typhimurium, les fimbriae *lpf*, *bcf*, *stb*, *stc*, *std*, et *sth* sont impliqués dans la persistance de l'infection intestinale par Typhimurium dans la souris (219). De plus, il a été démontré que les fimbriae *lpf*, *pef* et *csg* étaient nécessaires pour le développement d'un biofilm mature (125). Le fimbriae *lpf* permet aussi l'attachement aux cellules M des plaques de Peyer, au niveau du petit intestin (13). Typhi ne possède pas *lpf*, mais son attachement aux cellules intestinales est médié par le pili de type IVB (228).

Chez Typhimurium, seul l'opéron *fim* est exprimé dans des conditions de croissance *in vitro* (111). Par cytométrie en flux, à l'aide d'anticorps dirigé contre la sous unité majeure de chaque fimbriae, il a été démontré que plusieurs opérons fimbriaires de Typhimurium sont exprimés pendant l'infection intestinale de la souris (111). Les conditions *in vivo* semblent donc induire l'expression des fimbriae chez *Salmonella*.

Dernièrement, l'adhésine non-fimbriaire géante SiiE, codée sur le SPI-4, a été caractérisée. Elle est constituée de 53 domaines de type immunoglobuline et est requise pour l'attachement aux cellules épithéliales (76).

1.5. Régulation des facteurs de virulence

Les bactéries pathogènes intracellulaires facultatives comme *Salmonella* sont capables de survivre et de se multiplier non seulement à l'intérieur de l'hôte mais aussi à l'extérieur. Les bactéries rencontrent donc une diversité d'environnements stressants pour lesquels elles doivent s'adapter. De plus, certains facteurs de virulence comme les toxines et les SST3 ne sont apparemment d'aucune utilité à l'extérieur de l'hôte et ils ne sont généralement pas exprimés constitutivement. Également, un facteur de virulence donné n'est pas requis tout au long de l'infection, mais est souvent impliqué à une étape précise de l'infection tel que le SST3-1 et le SST3-2. En conséquence, les facteurs de virulence sont souvent régulés de façon précise et leur expression est contrôlée par plusieurs régulateurs qui réagissent à de nombreux signaux environnementaux (15). Quelques-uns des régulateurs de *Salmonella* impliqués dans l'expression des facteurs de virulence seront discutés ci-dessous (Fig. 3).

1.5.1. Système à deux composantes (TCS)

Les TCS sont ubiquitaires chez les procaryotes et permettent de détecter et de répondre aux stimuli rencontrés par la bactérie (15). Les TCS sont généralement composés d'un régulateur soluble et d'un senseur situé au niveau de la membrane qui possède une activité kinase/phosphatase. Lorsque le senseur détecte son signal, il y a autophosphorylation d'un résidu histidine. Ce groupement phosphate est ensuite utilisé pour phosphoryler un résidu d'acide aspartique au niveau du régulateur. Le régulateur subit alors un changement de conformation qui change ses propriétés de liaison à

l'ADN. Quelques exemples de TCS et leurs implications dans la régulation des facteurs de virulence de *Salmonella* seront abordés ci-dessous.

1.5.1.1. PhoP/PhoQ

Il a été proposé que le TCS PhoP/PhoQ pourrait agir comme un interrupteur moléculaire entre le phénotype d'invasion et celui de survie dans les macrophages (Fig. 3). En effet, PhoP/PhoQ permet l'induction de l'expression du SST3-2 en induisant l'expression du TCS SsrA/SsrB (19), mais réprime l'expression de SST3-1 en réprimant l'expression de *hilA* (7). PhoP/PhoQ est aussi impliqué dans la régulation de gènes impliqués dans la modification des LPS, dans la survie dans des conditions de concentration faible en magnésium (Mg^{2+}), dans la résistance aux PA, au pH acide et aux sels biliaires (82). En conséquence, la mutation de PhoP ou de PhoQ réduit dramatiquement la virulence de *Salmonella* dans la souris et diminue son taux de survie dans les macrophages (63, 149).

PhoQ est la composante senseur de ce système et est situé dans la membrane cytoplasmique. Il a été démontré que PhoQ détecte la présence des cations divalents Mg^{2+}, Ca^{2+} et Mn^{2+} (213). Une grande concentration de Mg^{2+} stimule la fonction phosphatase de PhoQ, ce qui diminue la phosphorylation de PhoP et par conséquent son activité (29, 154). Lorsque la concentration de Mg^{2+} devient faible, l'activité kinase de PhoQ est rétablie et PhoP devient phosphorylé. PhoP est alors en mesure d'activer ou de réprimer la transcription des gènes qu'il régule en se liant à des séquences répétées au niveau du promoteur (130). Le SST3-2 est positivement régulé par PhoP et est induit en

présence de concentration faible (de l'ordre du micromolaire) de Mg^{2+} (49). Or, la concentration de Mg^{2+} à l'intérieur des macrophages est de l'ordre du millimolaire (143). Si PhoP régule positivement le SST3-2 à l'intérieur des macrophages, mais que la concentration de Mg^{2+} est trop élevée pour activer la fonction kinase de PhoQ, c'est que PhoQ doit nécessairement détecter la présence d'une autre molécule. Dernièrement, il a été démontré que la liaison de PA à PhoQ déplace les atomes de Mg^{2+} et rétablit son activité kinase (6). Ainsi, même si la concentration de Mg^{2+} est élevée, la présence de PA permet d'activer PhoP et d'induire l'expression du SST3-2.

1.5.1.2. PmrA/PmrB

Le TCS PmrA/PmrB (Fig. 3) est nécessaire pour la résistance au PA et à la toxicité produite par le FeIII (183, 222). Il a été démontré que PmrB répond à la présence de concentration élevée de FeIII par la phosphorylation de PmrA (222). PmrA induit ensuite la transcription de *pmrE* et de *pmrF* (86). PhoP/PhoQ est capable d'activer PmrA via l'induction de la transcription de *pmrD* (114).

1.5.1.3. OmpR/EnvZ

Le TCS OmpR/EnvZ (Fig. 3) régule l'expression des porines OmpF et OmpC selon l'osmolarité. En condition de fortes osmolarité, OmpF est produite préférentiellement tandis qu'en condition de faible osmolarité, OmpC prédomine (203). Le changement d'osmolarité est détecté par EnvZ qui phosphoryle OmpR (109). OmpR permet alors l'expression des gènes qu'il régule, dont la capsule Vi (170). Il a aussi été démontré que

ompR était induit en condition de pH acide et que OmpR contrôle la réponse à l'acidité, mais indépendamment de EnvZ (10). OmpR/EnvZ est responsable de la régulation du SST3-2 en fonction de l'acidité et de l'osmolarité du milieu (126). De plus, ce TCS régule aussi l'expression du SST3-1 via HilD et HilC (136). L'acidité de l'estomac permettrait l'induction du SST3-1 et l'acidité de la vacuole permettrait l'expression du SST3-2 (184).

1.5.2. SlyA

En 1996, un groupe de recherche affirmait avoir découvert une nouvelle hémolysine, nommée SlyA, nécessaire à la survie de *Salmonella* dans les macrophages (132). Cette affirmation était basée sur deux observations. Premièrement, une souche Δ*slyA* survivait moins bien que la souche sauvage dans les macrophages et était moins virulente dans la souris. Deuxièmement, l'introduction de *slyA* dans *E. coli* rendait cette bactérie hémolytique sur gélose sang. Il a été démontré par la suite que *slyA* était requis pour la survie intracellulaire, mais pas pour l'invasion des cellules M des plaques de Peyer (45).

La fonction d'hémolysine de SlyA a été mise en doute lorsqu'il a été démontré que SlyA induisait en fait l'expression de ClyA dans *E. coli*, ce qui donnait le phénotype d'hémolyse (164). ClyA est une hémolysine qui forme un pore dans les membranes (137). SlyA semblait donc avoir un rôle au niveau de la régulation génique et l'expression de plusieurs protéines semblait être régulée par *slyA* (27, 198). En fait, SlyA est nécessaire à l'expression du SST3-2, ce qui explique la baisse de survie

intracellulaire observée chez la souche Δ*slyA* (133). Plusieurs gènes régulés par PhoP/PhoQ sont aussi régulés par SlyA, incluant le SST3-2 et plusieurs gènes impliqués dans la résistance aux PA (160). Il a été suggéré que SlyA puisse avoir une action de coactivateur, en étant activé par la liaison à un ligand encore inconnu, ou d'antirépresseur, en déplaçant un répresseur comme H-NS (160).

1.6. Méthode d'identification de gènes exprimés *in vivo*

L'identification des gènes induits par une bactérie pathogène dans des conditions d'infection permet d'améliorer les connaissances sur les mécanismes de pathogenèse et de découvrir de nouveaux facteurs de virulence. Ainsi, plusieurs méthodes ont été développées dans ce but. Celles qui sont le plus couramment utilisées seront discutées ci-dessous.

1.6.1. « *In Vivo* Expression Technology » (IVET)

La technique IVET est basée sur l'identification de promoteurs induits lors de l'infection et a été utilisée la première fois pour étudier Typhimurium dans le modèle murin de la fièvre typhoïde (141). Elle utilise un plasmide suicide qui code pour un gène essentiel (*purA*) et un gène rapporteur (*lacZ*) sans promoteur et une souche mutante pour le gène essentiel *purA*. Une banque de promoteur est construite en clonant des fragments de gènes en amont de *purA*. Ces promoteurs contrôlent donc l'expression de *purA* et de *lacZ*. La banque de plasmides est ensuite transformée dans la souche Δ*purA*. Il y aura alors recombinaison entre le promoteur contenu sur le plasmide et la région homologue

sur le chromosome. La synthèse de purine est essentielle pour la viabilité de la bactérie. La souche Δ*purA* peut pousser sur gélose dans laquelle on ajoute les purines, mais elle ne peut survivre *in vivo*. Les promoteurs activés *in vivo* permettront de transcrire *purA* et de complémenter la mutation, permettant alors la survie *in vivo*. Les bactéries récupérées post-infection contiendront donc, en amont de *purA*, un promoteur actif *in vivo*. Il est ensuite possible d'identifier les promoteurs spécifiquement actifs *in vivo* en sélectionnant pour les colonies qui n'expriment pas *lacZ*. Le séquençage de la région en amont de *purA,* permet d'identifier le promoteur, et par conséquent le gène induit lors de l'infection. Une variante de cette technique utilise le gène *cat* au lieu de *purA* qui permet de résister au chloramphénicol (142). L'animal modèle est traité au chloramphénicol, et les bactéries qui sont récupérées post-infection contiennent un promoteur situé en amont de *cat* qui est induit.

Cette technique a comme avantages majeurs d'être relativement facile à réaliser et de ne pas nécessiter d'appareil particulier ou de méthode coûteuse. Elle permet d'identifier des gènes induits lors de l'infection, pourvu que l'insertion du plasmide ne désactive pas un gène essentiel pour l'infection. Dans ce cas, la souche ne pourra pas être récupérée et le promoteur ne sera pas identifié, ce qui est un désavantage non négligeable. De plus, un promoteur activé transitoirement, par exemple au début de l'infection seulement, ne permettra pas de complémenter la mutation de *purA* tout au long de l'infection et ce promoteur ne pourra pas être identifié puisque la bactérie ne survivra pas. Enfin, cette technique ne permet pas d'identifier globalement tous les gènes induits *in vivo* puisqu'elle repose sur le séquençage des bactéries isolées post-infection.

Cette technique a été utilisée avec succès pour étudier diverses bactéries pathogènes. Elle a été utilisée pour étudier Typhimurium dans le modèle murin de fièvre typhoïde et des régulateurs (*phoP* et *pmrB*), des gènes impliqués dans le transport de métaux (*mgtAB*, *fhuA* et *entF*) et quelques gènes sans fonction connue ont été identifiés (36, 96). La technique a aussi été appliquée pour étudier *Klebsiella pneumoniae* dans un modèle murin (121), *Actinobacillus pleuropneumoniae* dans un modèle porcin (66) et *Escherichia coli* dans un modèle murin (117), entre autres.

1.6.2. « Signature-tagged mutagenesis » (STM)

La méthode STM a été mise au point pour pouvoir tester simultanément une grande quantité de mutants (98). Les mutants sont produits par l'insertion de transposons qui ont chacun une étiquette composée d'une séquence unique placée entre deux séquences connues, pour pouvoir amplifier l'étiquette par PCR. Ainsi, chaque mutant peut être identifié par immunobuvardage de Southern en utilisant la séquence de l'étiquette comme sonde. En comparant le profil d'hybridation de la banque de mutant avant et après infection, il est possible d'identifier les mutants qui n'ont pas survécu. Après clonage de la région d'ADN contenant le transposon, il est possible d'identifier le gène muté par séquençage. Cette technique a été utilisée pour la première fois pour trouver des mutants de Typhimurium incapable de survivre dans la souris (98). Plusieurs facteurs de virulence connus ont été identifiés, y compris des gènes du SPI-2, ce qui confirme l'utilité de la technique.

En théorie, cette technique peut identifier tous les gènes essentiels à la survie de la bactérie lors d'infection, à condition que la banque de mutant soit assez complexe. Par contre, puisque l'identification est basée sur la technique de l'immunobuvardage de Southern et du séquençage, qui ne sont pas des méthodes à haut débit, le nombre de gènes identifiés est limité. Pour palier à ces problèmes, STM a été utilisé en conjonction avec les biopuces (voir section 1.6.4) (190, 191). Cette variante, appelée TraSH, utilise des transposons contenant le promoteur pour la polymérase à ARN T7 dirigé vers l'extérieur. Il est donc possible de transcrire l'ADN entourant le transposon et de marquer l'ARN obtenu avec des fluorochromes. L'ARN marqué est ensuite hybridé aux biopuces. Le signal obtenu avec l'ADN de la banque de mutants récoltés avant et après infection est comparé pour identifier les gènes qui, lorsque mutés, ne permettent pas au mutant de survivre lors de l'infection. Cette méthode a été utilisée pour identifier les gènes nécessaires à l'infection de macrophages murins et à l'infection de la souris par Typhimurium (30). Elle a aussi été utilisée pour identifier les gènes nécessaires à l'infection persistante de la souris par Typhimurium (124). Ces deux études ont utilisé les lignées de souris BalB/c et 129sv respectivement (voir section 1.4).

1.6.3. « Selective Capture of Transcribed Sequences » (SCOTS)

La technique SCOTS a été développée pour identifier des gènes de *Mycobacterium tuberculosis* exprimés lors de l'infection de macrophages humains en culture (80). D'une façon générale, cette technique repose sur la capture spécifique des transcrits bactériens (Fig. 6). L'ARN total est d'abord isolé et converti en ADNc en utilisant des amorces étiquettes (conserved primer). La partie 3' est un nonamère

aléatoire tandis que la partie 5' est une séquence connue qui n'a pas d'homologie dans le génome des espèces à l'étude et qui sert à étiqueter les transcrits. Ainsi, la conversion de l'ARN en ADNc se fait de façon aléatoire et ajoute la séquence étiquette à chacun des transcrits. Les transcrits peuvent ensuite être amplifiés par PCR en utilisant comme amorce la séquence étiquette. Une fois que l'ADNc est synthétisé, il s'agit de capturer les transcrits bactériens et de se débarrasser des transcrits eucaryotes et des transcrits bactériens codant pour l'ARN ribosomal (ARNr). Pour y arriver, de l'ADN génomique (ADNg) de la bactérie est biotinylé et pré-hybridé (bloqué) avec un excès d'ADN ribosomal cloné (ADNr). Vient ensuite l'étape de capture, où l'ADNg ainsi préparé est hybridé avec les ADNc provenant de l'infection. Seuls les ADNc bactériens pourront s'hybrider à l'ADNg. Les ADNc codant pour l'ARNr ne seront pas récupérés puisque les zones homologues sur l'ADNg sont déjà occupées par l'ADNr. Les hybrides ADNc-ADNg sont ensuite récupérés à l'aide de billes magnétiques couvertes de streptavidine. Les ADNc sont ensuite élués et amplifiés par PCR. Deux autres rondes de capture sont par la suite effectuées pour s'assurer de maximiser la diversité au niveau des transcrits bactériens et pour se débarrasser des transcrits eucaryotes et de ceux codant pour l'ARNr. Les ADNc ainsi obtenus sont alors clonés et séquencés, permettant donc d'identifier les gènes bactériens exprimés pendant l'infection.

Il est aussi possible d'enrichir les ADNc obtenus par SCOTS. La méthode d'enrichissement consiste à effectuer un second SCOTS en commençant avec les ADNc obtenus du premier SCOTS. Par contre, pour le second SCOTS, l'ADNg sera bloqué avec, par exemple, les ADNc obtenus par SCOTS à partir d'une autre condition. Ainsi, pour identifier des gènes exprimés seulement *in vivo*, les ADNc obtenus à partir d'une

condition *in vitro* devront être utilisés. Cette approche a été utilisée pour identifier des gènes de Typhi exprimés différemment dans les macrophages humains et dans le milieu de culture des macrophages (44). Dozois *et coll.* (54) ont utilisé la technique d'enrichissement pour identifier les gènes d'une souche d'*E. coli* pathogène aviaire exprimé pendant l'infection de poulet, mais absent dans le génome d'une souche de *E. coli* K-12 incapable de causer d'infection chez le poulet. Dans cette étude, l'ADNg de *E. coli* pathogène aviaire a été bloqué avec l'ADNg de *E. coli* K-12. Une qualité appréciable de SCOTS est sa grande versatilité, ce qui permet de l'utiliser pour répondre à des questions diverses comme celles décrites ci-dessus.

L'avantage majeur de SCOTS est que cette technique ne requiert pas de modification génétique de la bactérie étudiée, contrairement à IVET et à STM. De plus, elle ne requiert pas de modification des modèles d'infection utilisés habituellement, puisque la quantité de bactéries requise est faible. Par contre, elle ne permet pas d'obtenir une vue globale des facteurs de virulence exprimés pendant l'infection puisque l'identification est basée sur le séquençage des ADNc clonés. Une alternative possible pour contourner ce problème serait d'hybrider les ADNc obtenus par SCOTS à des biopuces pour identifier globalement les gènes exprimés pendant l'infection. Cette méthode a été utilisée pour identifier les gènes de *Helicobacter pylori* exprimés pendant l'infection de la muqueuse gastrique (81). Les ADNc obtenus après capture ont été hybridés à des biopuces sur membrane. Bien que ce type de biopuces ne permet qu'une analyse qualitative des résultats, cette étude a démontré l'efficacité de capture et d'amplification de SCOTS, puisque 70 % des gènes étaient détectés.

1.6.4. Biopuces

Toutes ces méthodes ont leurs avantages et leurs inconvénients, mais une seule, la méthode des biopuces, permet d'obtenir un portrait global des gènes induits lors de l'infection. Cette méthode permet la quantification relative des transcrits exprimés. Tous les gènes de la bactérie à l'étude sont préalablement déposés sur une lame de verre et servent de sonde. Le principe de base est l'hybridation compétitive sur cette lame des transcrits obtenus d'une condition test et d'une condition contrôle marqués avec des fluorochromes différents. Le signal des transcrits hybridés à chaque sonde est ensuite quantifié et analysé. Initialement, la technologie des biopuces a été appliquée au monde bactérien pour étudier l'expression génique dans des conditions *in vitro* qui mime des conditions environnementales retrouvées pendant l'infection (38). Par exemple, la réponse globale de Typhi au stress oxydatif produit par le peroxyde d'hydrogène a été étudiée (173). Par contre, il y a très peu d'études de transcriptome avec des bactéries pathogènes dans des conditions *in vivo* puisque les biopuces requièrent l'obtention d'une grande quantité d'ARN de très bonne qualité exempte de contamination d'ARN eucaryote (101). Ceci est compliqué par la nature instable de l'ARN bactérien et par la rapidité à laquelle les bactéries adaptent leur profil d'expression aux nouvelles conditions rencontrées (38). De ce fait, l'ARN doit être rapidement stabilisé pendant l'extraction, ce qui s'avère difficile dans le cas de bactérie intracellulaire (38). De plus, l'ARN bactérien est composé en grande partie d'ARNr (85%) et l'ARN eucaryote est beaucoup plus abondant que l'ARN bactérien dans des conditions d'infection (38). Ceci a pour effet de diminuer considérablement le signal obtenu après hybridation aux biopuces.

Pour contrer ces problèmes, l'ARN peut être obtenu à partir de conditions qui minimisent le contact avec les cellules de l'hôte et qui favorisent une grande récupération de bactéries. Dans ces conditions, une bonne quantité d'ARN bactérien peut être extraite tout en diminuant significativement la contamination par l'ARN eucaryote. Ainsi le transcriptome de *Vibrio cholerae* a été caractérisé en isolant l'ARN de selles de patients infectés (148) et celui de *E. coli* uropathogène (UPEC) a été obtenu à partir de bactéries présentes dans l'urine de souris infectées (197). Bien que ce genre d'approches permettent d'identifier de nouveaux facteurs de virulence, certains problèmes au niveau de la pertinence biologique de la condition choisie peuvent être soulevés. Dans les deux cas, les bactéries qui sont présentent dans l'urine ou dans les selles se sont détachées de l'épithélium de l'hôte. Ainsi, leur profil d'expression peut être différent de celui des bactéries qui sont en contact intime avec les cellules de l'hôte. Le même genre d'objection peut être soulevé dans le cas où l'ARN est obtenu à partir de bactéries situées dans des chambres à dialyse implantées dans l'animal modèle. Cette méthode a été utilisée pour obtenir le transcriptome de *Borrelia burgdorferi* (181). Dans ce cas, les bactéries ont seulement accès aux molécules solubles de l'hôte. Ainsi, le transcriptome reflète la réponse bactérienne à la présence de ces molécules et non au contact des cellules de l'hôte. Cette approche a aussi été utilisée pour comparer l'expression de systèmes de transport du fer de *Staphylococcus aureus* dans des conditions de concentration faible en fer *in vitro* et dans des chambres à dialyse *in vivo* (3).

Pour étudier le transcriptome de Typhimurium dans les macrophages murins, la méthode de lyse différentielle a été développée (58). Il s'agit dans un premier temps de lyser les cellules de l'hôte sur glace dans une solution qui stabilise l'ARN. Les bactéries sont ensuite récupérées et lysées à leur tour pour en extraire l'ARN qui est exempt d'ARN eucaryote. Par contre, même avec cette méthode, très peu d'ARN bactérien peut être récupéré. Les chercheurs ont donc eu recours à une quantité élevée de cellules en culture, une forte dose d'infection (100 bactéries par cellule) et ont dû opsoniser les bactéries pour optimiser leurs entrées dans les phagocytes. Ainsi, bien que les bactéries soient dans ce cas en contact intime avec les cellules de l'hôte, comme lors d'une véritable infection, la forte dose d'infection ne reflète pas les conditions normales d'infection. De plus, il est impossible d'appliquer cette méthode à des conditions où le matériel biologique est limité. Dans le cas d'une bactérie pathogène pour laquelle un modèle animal est utilisé, il est toujours possible de combiner l'ARN obtenu de plusieurs animaux. Ce genre d'approche a été utilisé pour obtenir le transcriptome de *Mycobacterium tuberculosis* lors de l'infection pulmonaire de la souris (202). Cent souris infectées ont été nécessaires pour chaque temps étudié, ce qui s'avère laborieux, coûteux et éthiquement discutable.

Pour augmenter la quantité de transcrits bactériens, une alternative possible serait d'utiliser une méthode d'amplification. Dans le cas d'études portant sur le transcriptome eucaryote, les transcrits sont généralement amplifiés grâce à la polymérase à ARN T7 en utilisant des amorces poly dT, puisque les transcrits eucaryotes sont polyadénylés (189). Malheureusement, les transcrits bactériens sont généralement peu ou pas du tout polyadénylés et il est donc impossible d'utiliser directement cette approche. Il est

envisageable d'ajouter une séquence poly A aux transcrits bactériens pour pouvoir les amplifier de cette façon. Par contre, les transcrit eucaryotes seront amplifiés eux aussi, ce qui ne changerait rien au problème.

1.7. Problématique et objectifs

Le modèle murin de la fièvre typhoïde est utilisé pour identifier les gènes nécessaires à l'infection systémique par *Salmonella*, comme expliqué à la section 1.4. En fait, ce modèle permet d'étudier les gènes nécessaires à l'infection systémique de la souris par Typhimurium. Ce sérovar cause chez l'homme une infection localisée qui est bien différente de l'infection systémique causée par Typhi. La capacité de *Salmonella* à causer une infection systémique semble être reliée à la capacité de survivre dans les macrophages de l'hôte. Ainsi, il a été démontré que Typhi survit mieux dans les macrophages humains tandis que Typhimurium survit mieux dans les macrophages murins (193). Cette différence pourrait être expliquée par l'utilisation de facteurs de virulence spécifiques à chacun de ces sérovars. Cette possibilité est appuyée par le séquençage du génome de Typhi et de Typhimurium qui a permis d'identifier 601 gènes uniques à Typhi (146, 167).

L'hypothèse principale de cette thèse est que les gènes supplémentaires de Typhi (absent de Typhimurium) pourraient être impliqués dans la capacité de Typhi d'infecter l'homme de façon systémique. Puisque l'étape de survie dans les macrophages semble être cruciale pour l'établissement d'une infection systémique, les gènes uniques de

Typhi, exprimés lors de l'infection de macrophages humains, pourraient être des facteurs de virulence.

L'objectif premier de cette thèse est donc d'identifier les gènes uniques de Typhi exprimés lors de l'infection de macrophages humains. Nous avons dans un premier temps utilisé SCOTS pour capturer les gènes uniques de Typhi (absent chez Typhimurium) exprimés lors de l'infection de macrophages humains. Cette méthode n'étant pas exhaustive, comme expliqué à la section 1.6.3, nous avons voulu, dans un deuxième temps, utiliser les biopuces pour identifier globalement les gènes exprimés par Typhi durant l'infection de macrophages. Par contre, la technique des biopuces requiert une grande quantité de transcrits, comme expliqué à la section 1.6.4. Malheureusement, dans les conditions normales d'infection de macrophages humains, la quantité de transcrits bactériens disponibles est trop faible pour permettre l'utilisation de biopuces. La technique SCOTS permet d'amplifier les transcrits bactériens tout en rejetant les transcrits eucaryotes et ne demande pas de modification au modèle d'infection couramment utilisé (80). L'utilisation de SCOTS en conjonction avec les biopuces devrait permettre de résoudre les problèmes mentionnés ci-haut.

L'objectif second de cette thèse est d'identifier de nouveaux facteurs de virulence propres à Typhi. Les gènes uniques à Typhi exprimés dans les macrophages pourraient être impliqués dans la virulence de Typhi et dans son interaction avec les macrophages humains. L'implication de certains des gènes identifiés sera caractérisée. Le choix des gènes à étudier sera basé sur leur homologie à des facteurs de virulence connus et sur leur profil d'expression. L'implication de ceux-ci dans la pathogenèse de

Typhi sera d'abord étudiée en les retirant du génome et en évaluant les interactions des souches ainsi mutées avec les macrophages humains, comparées à la souche sauvage.

1.8. Figures

Figure 1 : Pathogenèse de Typhi

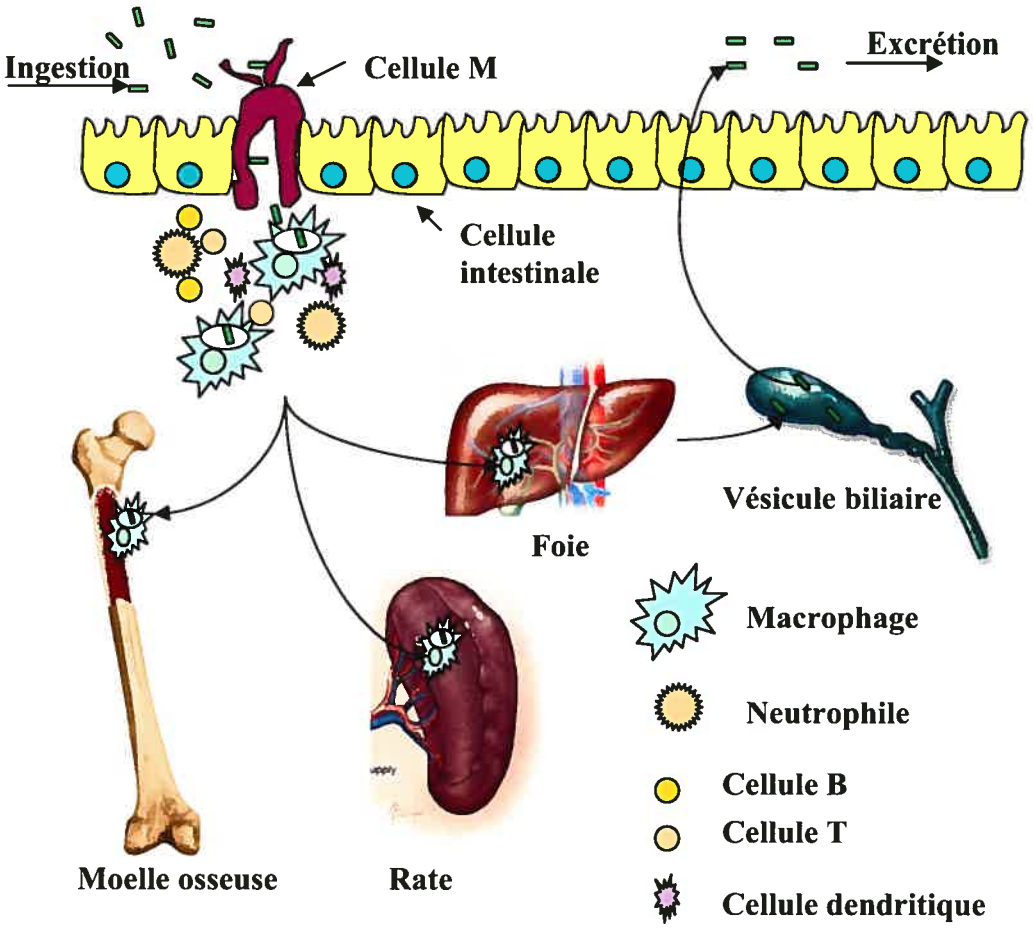
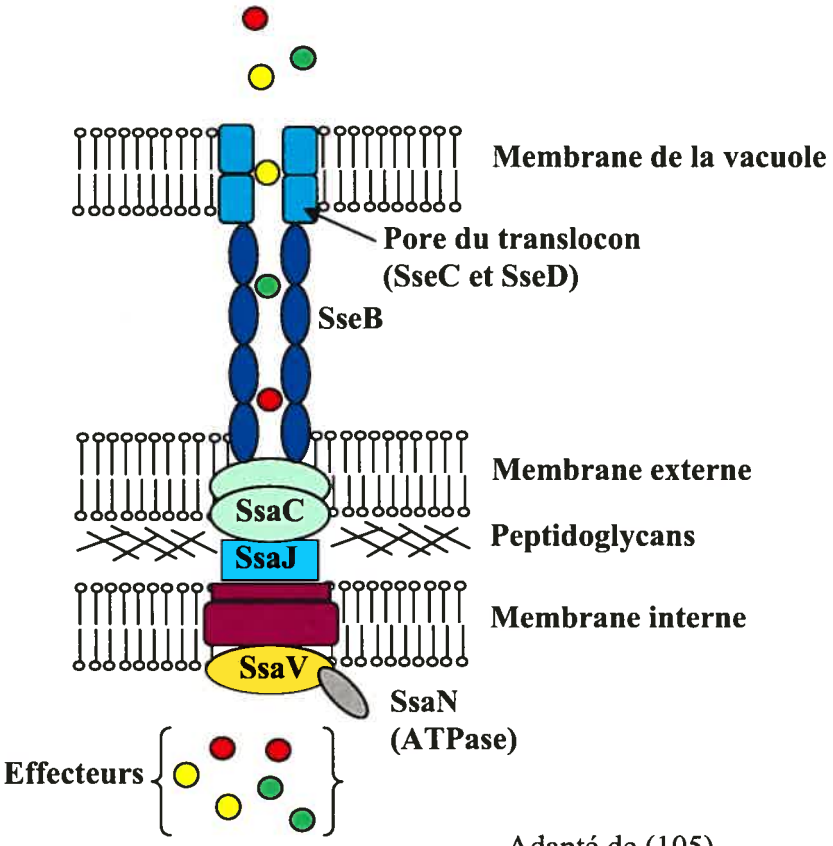
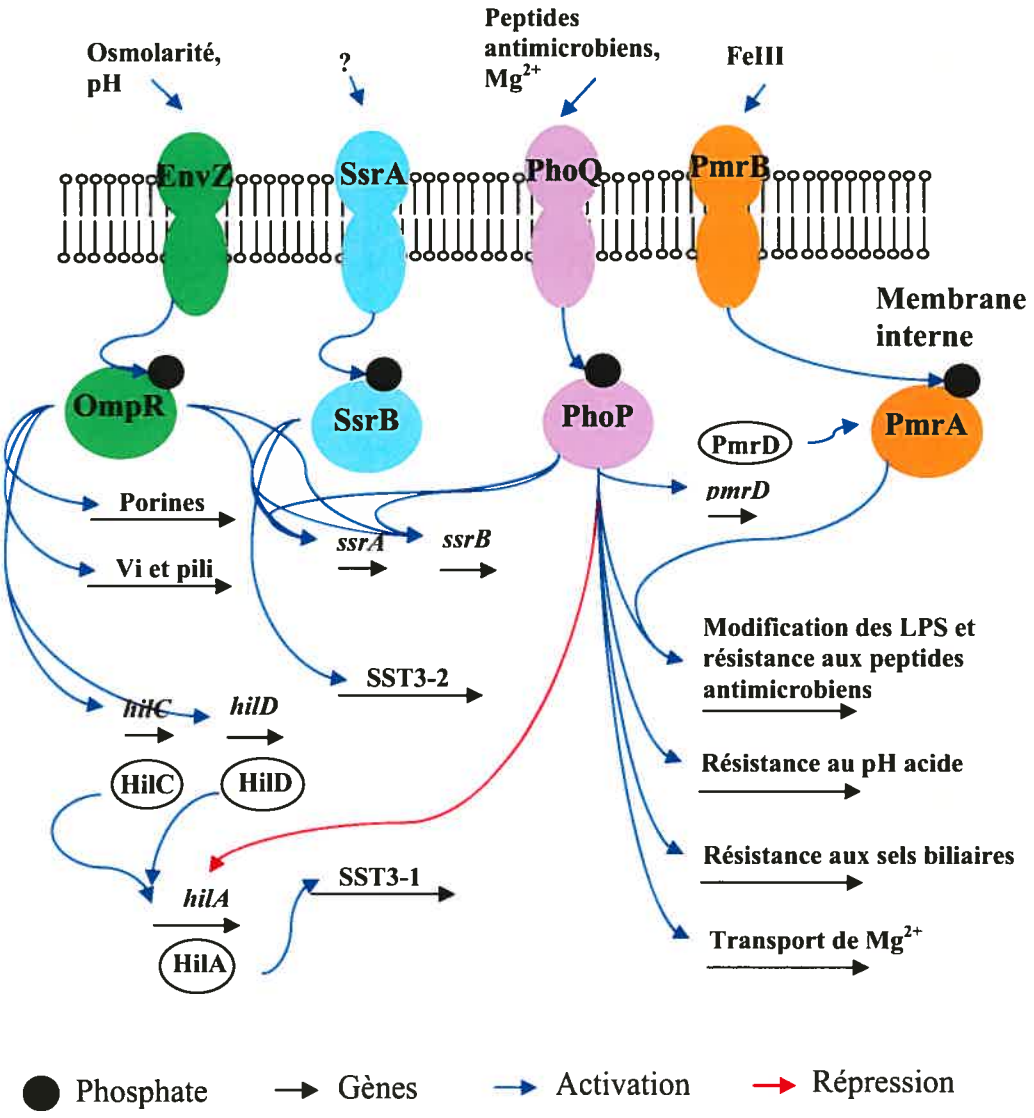


Figure 2 : Système de sécrétion de type trois (SST3) impliqué dans la survie dans les macrophages.



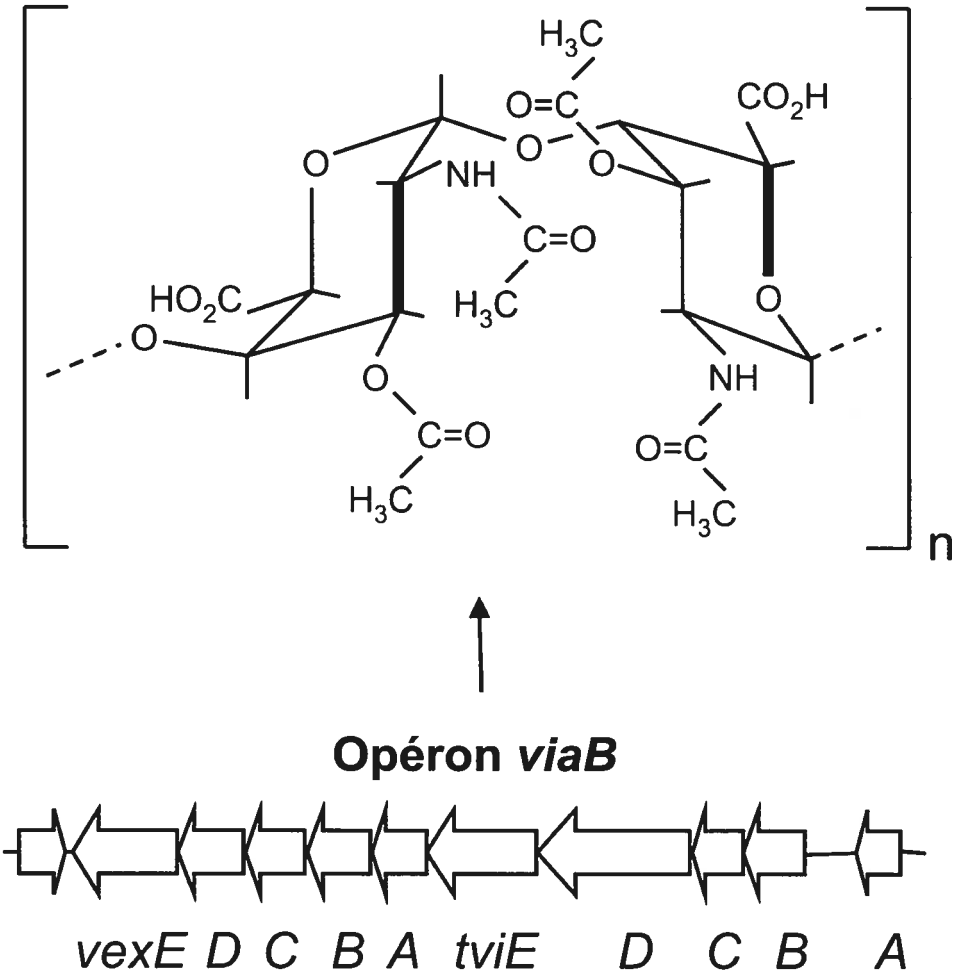
Adapté de (105)

Figure 3 : Quelques systèmes à deux composantes de *Salmonella*.



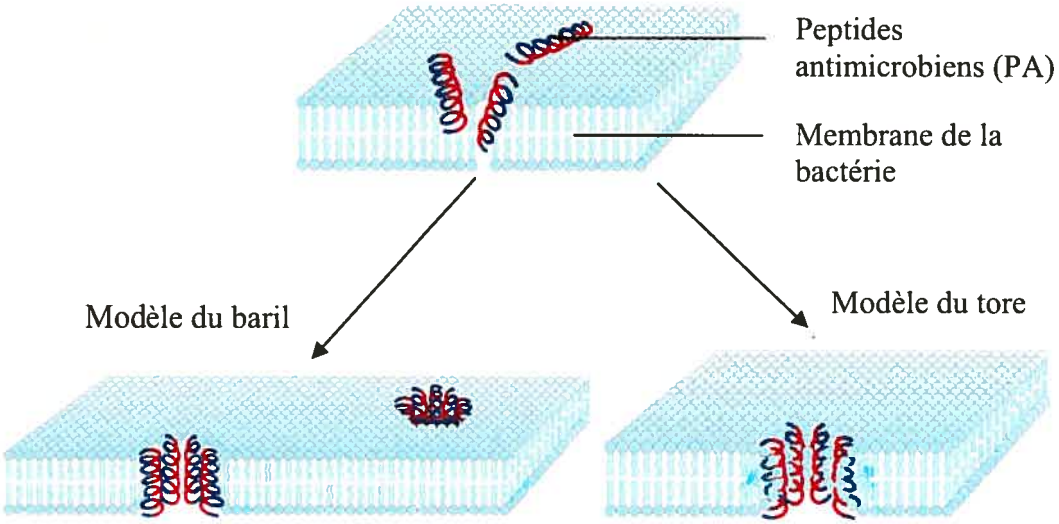
Adapté de (15)

Figure 4 : Composition de la capsule Vi.



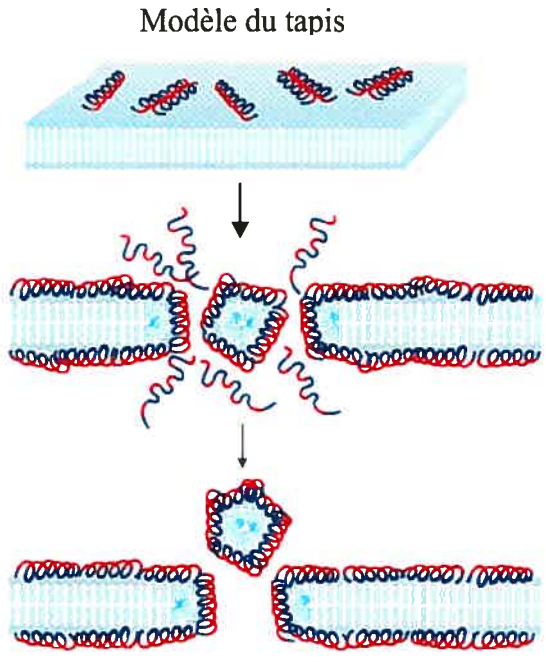
Adapté de (176)

Figure 5 : Modèles de l'interaction des peptides antimicrobiens avec la membrane bactérienne.



Les PA s'insèrent dans la membrane. Le pore est formé par les PA.

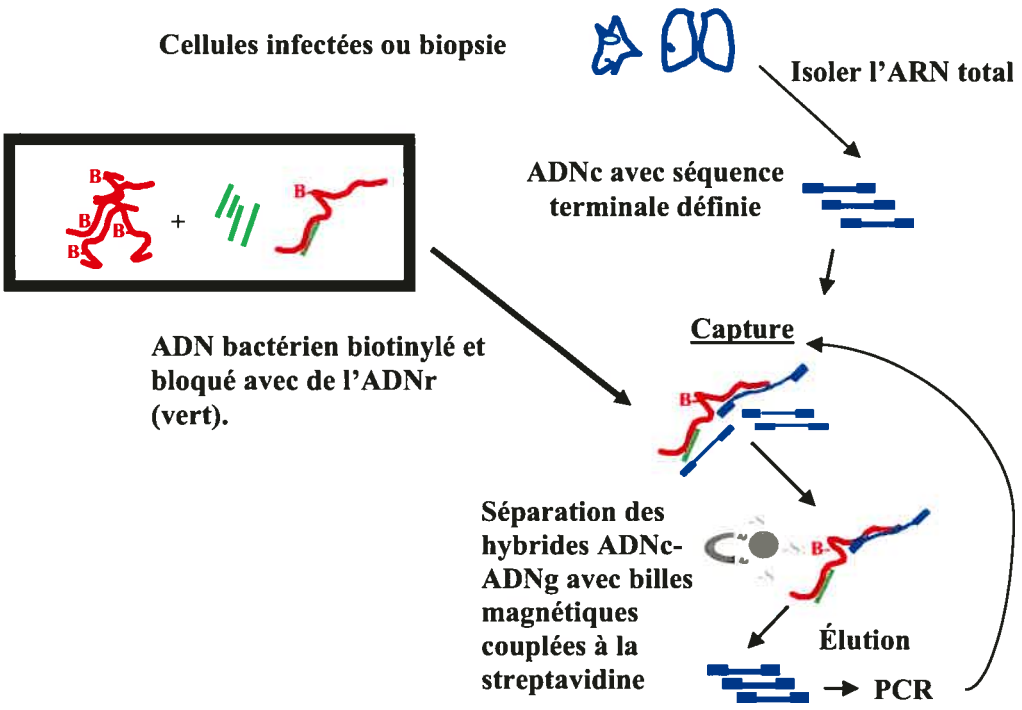
Les PA induisent le repliement de la membrane. Le pore est formé par les phospholipides.



Les PA couvrent la surface de la membrane.

Adapté de (25)

Figure 6 : Selective Capture Of Transcribed Sequences (SCOTS)



Adapté de (80)

Préface au Chapitre 2

La première approche utilisée afin d'identifier des gènes uniques à Typhi exprimés lors de l'infection de macrophages humains est basée sur l'utilisation de la méthode SCOTS avec une étape d'enrichissement. Pour plus de clarté, la procédure expérimentale utilisée est schématisée à l'annexe A1. Cette application de SCOTS a permis d'identifier 36 gènes uniques à Typhi, exprimés lors de l'infection. Ces résultats ont été publiés dans le journal « Infection and Immunity » sous forme de note. De ce fait, le corps de l'article n'est pas divisé en section. Les résultats contenus dans la figure 2 ont été analysés par la suite avec le logiciel Tigr MeV pour produire une figure en couleur qui permet une meilleure visualisation des résultats (annexe A2).

Les expériences décrites dans cet article de même que l'écriture du manuscrit ont été effectuées à parts égales par moi et France Daigle. Les fonds nécessaires à ce projet ont été fournis Roy Curtiss III et France Daigle.

CHAPITRE 2, 1er article

Selective capture of *Salmonella typhi* genes expressed in macrophages that are absent from the *Salmonella typhimurium* genome

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Running title: *S. typhi*-specific genes expressed within macrophages

Keys Words: *Salmonella*, SCOTS, *in vivo* gene expression, pathogenesis

ABSTRACT

Thirty-six *Salmonella typhi*-specific genes, absent from the *S. typhimurium* genome, that were expressed in human macrophages, were identified by selective capture of transcribed sequences (SCOTS). These genes are located on 15 unique loci of the *S. typhi* genome, including pathogenicity islands (SPI-7, SPI-8 and SPI-10) and bacteriophages (ST15, ST18 and ST35).

TEXT

Salmonella enterica is composed of more than 2400 serovars that can infect humans and a great diversity of mammals, birds, and reptiles (17). Some *Salmonella* serovars are closely related genetically but differ in their host range. For example, *Salmonella enterica* serovar Typhi (*S. typhi*) is the etiologic agent of typhoid fever, and is a host-adapted serovar that is specific for humans, whereas *S. enterica* serovar Typhimurium (*S. typhimurium*) is a broad-host range pathogen. Since there is no animal model to study *S. typhi* pathogenicity, little is known about the specific factors contributing to its ability to cause typhoid fever and its adaptation to the human host. Consequently, what is known about *S. typhi* pathogenicity has been largely extrapolated from studies of *S. typhimurium* infections in mice. Although *S. typhi* and *S. typhimurium* serovars share many virulence properties, each serovar causes a distinct type of disease in humans. *S. typhi* is associated with systemic infections, whereas *S. typhimurium* is usually associated with a localized gastroenteritis. The complete genome sequences of *S. typhi* strains CT18 and Ty2 and *S. typhimurium* strain LT2 are now available (7, 21, 23).

There are significant differences between *S. typhi* and *S. typhimurium*, and unique regions of *S. typhi* DNA are scattered along the chromosome. Such unique regions can be single genes or groups of gene blocks (up to 100 genes). There are 601 genes (13.1 % of the *S. typhi* genome) in 82 regions that are unique to *S. typhi* CT18 compared with *S. typhimurium* LT2 and 479 genes (10.9 % of the *S. typhimurium* genome) that are unique to *S. typhimurium* compared to *S. typhi* (21). Gene clusters unique to particular bacteria are likely to contribute to adaptation to particular environments or host niches, and may contribute to pathogenicity. Thus, we can consider that *S. typhi* possesses unique genetic information that may be important for systemic spreading and survival in the human host.

As survival within macrophages is an essential step for *Salmonella* pathogenesis (10), we have used selective capture of transcribed sequences (SCOTS) (6, 14) to identify genes expressed by *S. typhi* within macrophages (6, 14). In order to obtain sequences unique to *S. typhi* (absent from the *S. typhimurium* genome), we have used an additional strain-specific enrichment step as was previously described to identify *in vivo* expressed pathogen-specific genes from avian pathogenic *E. coli* (8). The human macrophage-like cells, derived from the monocyte cell line THP-1 (ATCC TIB-202), were infected with *S. typhi* ISP1820 as described previously (5, 6). The infected monolayers were lysed 2 h post-infection by addition of TRIzol (Invitrogen) and RNA was prepared according to the manufacturer's instructions. A 5 µg sample of RNA was isolated from infected macrophages or other growth conditions, was treated with RNase-free DNase, and was reverse transcribed by random priming (6, 11). Three rounds of bacterial cDNA capture were done as described previously (5, 6, 14). The cDNA

mixtures obtained following SCOTS were used as probe templates or for competitive hybridization enrichment. The cDNA enrichment for *S. typhi*-specific sequences was obtained by using an excess (10 μg) of genomic DNA from *S. typhimurium* strain SL1344 as described previously (8). cDNAs were then cloned after 3 rounds of this competitive hybridization enrichment using the Original TA Cloning® kit (Invitrogen) according to the manufacturer's instructions. Cloned inserts were sequenced at our sequencing facility. Database searches and DNA and protein similarity comparisons were carried out with the BLAST algorithms (1) available from the National Center for Biotechnology Information (NCBI). We sequenced 60 clones, corresponding to 45 different genes and 36 of these clones corresponded to distinct genes that are specific to *S. typhi*.

***S. typhi*-specific genes**. We identified 36 genes expressed intracellularly by *S. typhi* that are absent from *S. typhimurium* (Table 1). The genomic location of the 36 genes was analyzed and 15 distinct regions, ranging from 0.8 kb to 134 kb, containing at least 2 *S. typhi*-specific genes per locus were identified (Table 1). SCOTS identified serovar Typhi-specific regions as small as 0.8 kb in length. Blast comparisons with microbial genomes (including unfinished genomes) at NCBI (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) were also performed to verify the presence of the regions in ten *Salmonella* strains from different serovars that have been or are currently being sequenced. Strains in the genome databases included *S. typhi* strains CT18 (7) and Ty2 (21); *S. typhimurium* strains LT2 (23), DT104 (Sanger

Institute), and SL1344 (Sanger Institute); *S. dublin* (University of Illinois at Urbana-Champaign); *S. enteritidis* LK5 (University of Illinois at Urbana-Champaign); *S. bongori* 12419 (Sanger Institute); *S. paratyphi* A strain ATCC9150 (Washington University), and *S. paratyphi* B strain SPB7 (Washington University). Seven regions were present only in both *S. typhi* strains and 5 regions were present in *S. typhi* and *S. paratyphi* (Table 1). An extensive data set of gene distributions among a diversity of 79 *Salmonella* strains (25) was also used to determine the distribution of the genes identified by SCOTS in *Salmonella* species (Table 1).

Hybridization profiles. Expression of the identified genes in other growth conditions was verified. *S. typhi* cDNAs were obtained by 3 rounds of SCOTS from bacteria grown in vitro in Luria-Bertani (LB) broth (log phase), or overnight in tissue culture medium (complete RPMI), or following infection of THP-1 human macrophages for 2 h and 24 h (T2, T24) or RAW264.7 murine macrophages for 2 h and 24 h (R2, R24). These cDNAs were then used as probes against the 36 *S. typhi*-specific genes by Southern blotting, using Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). The hybridization signals for each gene product were quantified by densitometry. SCOTS-cDNA was previously used as a probe hybridized to a membrane array to investigate global gene transcription by *Helicobacter pylori* (15). The hybridization results from the different experiments were clustered together using default parameters of Hierarchical clustering (HCL) from TIGR MeV (27) (Fig. 1). The hybridization profile experiments using infection of murine macrophages (R2 and R24) were closer to the in vitro experiments (LB and RPMI) than to the experiments using human macrophages (T2 and T24) (Fig. 1). This could be because of the host-restricted

phenotype of *S. typhi* or because only *S. typhi*-specific genes were analyzed or because many of the *S. typhi* genes expressed in human macrophages are repressed both in LB and murine macrophages. Three major hybridization profiles for the *S. typhi*-specific genes were obtained: 1) 14 genes exhibited a weaker hybridization signal when using the cDNA probes from infected murine macrophages (Fig. 2A); 2) 11 genes exhibited an equal signal in both macrophages (Fig. 2B); and 3) 6 genes exhibited a weaker hybridization signal when using the T2 and T24 cDNA probes from infected human THP-1 macrophages (Fig. 2C). Hybridization patterns of the five remaining genes (STY0207, STY1635, STY2026, STY2732, and STY3695) did not correspond to the three main hybridization profiles.

SPIs and prophages. Among the *S. typhi*-specific sequences, 25 genes were located on *Salmonella* pathogenicity islands (SPIs) or prophage-like elements (Table 1). It is clear that pathogenicity islands such as SPIs (20) as well as phage (2) contribute to strain- or serovar-specific genomic differences, and the evolution and virulence of bacterial pathogens. Sixteen of the *S. typhi*-specific genes identified are located on SPIs 7, 8 or 10. SPI-7 is a 134 kb region of mosaic structure (22, 24) and 14 identified genes are located on SPI-7. Seven of these genes belong to a putative DNA transfer system (Table 1) and 2 genes, STY4654 (*vexB*) and STY4656 (*tviE*), are part of the *viaB* locus, which encodes the Vi capsule. The Vi capsule seems to play a role in reducing early inflammatory responses from intestinal epithelial cells during infection with *S. typhi* (28). Similarly, the *viaB* locus may also influence the macrophage host response, as Vi contributes to survival of *S. typhi* within human macrophages (16). We observed a weaker hybridization signal for the *vexB* and *tviE* from cDNA probes derived from *S.*

typhi infected murine macrophages than in cDNA probes obtained from infected human macrophages (Fig 2A). Thus, the Vi capsule may play a role in host-specificity. In our previous studies, we have also identified genes *tviB* and *vexA* of the *viaB* locus, to be expressed in human macrophages (5). Hybridization signals for SPI-7 genes were always detected in human macrophages (Fig. 2A-B). We identified 10 genes encoded by four different prophage-like elements, ST15, ST18, ST35 and $SopE_{ST}$. Five of these genes were located on ST18, including STY2005, which codes for a hypothetical protein with a conserved GGDEF domain. The GGDEF domain is involved in signal transduction of virulence genes (12) and represents a protein family involved in the regulation of the production of cellulose and biofilms in *S. typhimurium* (13). The bacteriophage $SopE_{ST}$ is located within SPI-7 (see above) and one gene, *apI*, was identified by SCOTS. In bacteriophage 186 of *E. coli*, *apl* encodes a stress response protein that is both a repressor of *c*I and an excisionase (26). This may reflect that inside the macrophages, bacterial as well as lysogenic phage or phage-related genes respond to many stress conditions. It was previously demonstrated that phage induction in vivo was necessary for expression of virulence factors encoded by phage such as the Shiga toxin (32, 33).

Fimbriae- and pili-encoding genes. *Salmonella* contain a number of putative fimbriae- and pili-encoding systems (19). However, overall, little is known about function or the conditions under which these putative structures are expressed, their role in virulence and their possible relationship to bacterial host-adaptation. By using SCOTS, we have identified genes encoding two putative fimbriae, Sta and Stg, and a type IV pilus system expressed inside human macrophages. STY3920 (*stgC*) contains a

premature stop codon that disrupts the expected ORF encoding for the usher and is therefore considered a pseudogene in *S. typhi* strain ISP1820 (data not shown) and other *S. typhi* strains (31). The Sta and Stg fimbriae were present in all *S. typhi* strains tested, and demonstrated a scattered distribution within *Salmonella* serovars (Table 1). The type IV pili encoded by the *S. typhi pil* operon is located on SPI-7 (see above). Type IV pili of *S. typhi* facilitate bacterial entry into human intestinal epithelial cells and mediate binding to the host cell cystic fibrosis transmembrane conductance regulator. The STY4539 (*pilL*) gene did not hybridize with cDNA probes from inside murine macrophages (Fig. 2A). Some adhesins, such as thin aggregative fimbriae encoded by the *agf* (*csg*) genes, and plasmid-encoded fimbriae (Pef), were previously shown to be expressed during *S. typhimurium* infection of macrophages (9). It is currently unknown whether production of either fimbriae or pili by an intracellular pathogen such as *S. typhi* when inside host cells confers a specific advantage for virulence or host-specificity.

Other unique regions. Two putative transposases, STY0115 and STY4848 (SPI-10) were identified by SCOTS. These two transposases were detected constitutively (Fig. 2B). In *S. typhimurium*, some transposases such as *tnpA*, STM1860 and STM2904, are also expressed intracellularly (9). Regulation of transposases is not well understood to date and their expression may reflect the bacterial growth state (4). However, a putative transposase, *gipA*, encoded on phage Gifsy-1 of *S. typhimurium*, enhances growth or survival of *S. typhimurium* in the Peyer's patches of the murine small intestine (29). Putative regulatory proteins encoded by STY3845 and STY4412, encoding for a putative regulator related to CopG and DeoR respectively, were identified. STY4221 is located on a 6 kb region and encodes a putative aminotransferase

that shows identity with a hemolysin of *Treponema denticola* (3). We have identified two out of three ORFs (STY3948 and STY3950) in a 3 kb region that encodes hypothetical proteins that are conserved in some *Vibrio spp.* In *S. typhi*, this locus is inserted in the region corresponding to the galactonate, *dgo* operon, in *S. typhimurium* which is absent from the *S. typhi* genome.

Despite the powerful genomic and bioinformatic tools available to us, we currently have a limited understanding of the molecular basis of infectious diseases. The availability of complete sequences of numerous bacterial pathogens or serovars combined with functional genomics will be invaluable in to further understand the in vivo expression of bacterial genes and mechanisms of bacterial pathogenesis. Genome comparison of *Salmonella* serovars Typhi and Typhimurium identified 601 *S. typhi*-specific genes (23). In this report, we were able to determine that 36 *S. typhi*-specific genes were expressed during macrophage infection. The majority of the *S. typhi*-specific genes were located on SPIs and phages, which is not surprising, as these elements are horizontally acquired or transferred (2, 20). Some of the putative proteins identified in this study, that are located on SPIs, phages or chromosome, may represent new effector proteins or fitness factors. In *S. typhimurium*, several type III effector proteins are encoded by prophages, such as SopE, or encoded by SPIs, such as SipA-D (SPI-1), SseA-G (SPI-2), or are located on small regions disperse throughout the chromosome, such as SopA, SopD and SifA (18, 34, 35). Further characterization is needed to determine the contribution of these *S. typhi*-specific genes to pathogenesis. By using SCOTS, we have been able to identify in vivo expressed genes unique to a specific pathogen. This kind of data can not be obtained with the sole comparison of genome and

this is why functional genomic approaches such as SCOTS represent a very powerful tool to better understand bacterial pathogenesis.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-410.
2. **Brussow, H., C. Canchaya, and W. D. Hardt.** 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol. Mol. Biol. Rev. **68:**560-602.
3. **Chu, L., A. Burgum, D. Kolodrubetz, and S. C. Holt.** 1995. The 46-kilodalton-hemolysin gene from *Treponema denticola* encodes a novel hemolysin homologous to aminotransferases. Infect. Immun. **63:**4448-4455.
4. **Craig, N. L.** 2004. Transposition. *In* F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology. ASM Press.

5. **Daigle, F., J. E. Graham, and R. Curtiss, 3rd.** 2001. Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). Mol. Microbiol. **41:**1211-1222.

6. **Daigle, F., J. Y. Hou, and J. E. Clark-Curtiss.** 2002. Microbial gene expression elucidated by selective capture of transcribed sequences (SCOTS). Meth. Enzymol. **358:**108-122.

7. **Deng, W., S. R. Liou, G. Plunkett, 3rd, G. F. Mayhew, D. J. Rose, V. Burland, V. Kodoyianni, D. C. Schwartz, and F. R. Blattner.** 2003. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. J. Bacteriol. **185:**2330-2337.

8. **Dozois, C. M., F. Daigle, and R. Curtiss, 3rd.** 2003. Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic *Escherichia coli* strain. Proc. Natl. Acad. Sci. U S A **100:**247-252.

9. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Microbiol. **47:**103-118.

10. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. (USA) **83:**5189-5193.

11. **Froussard, P.** 1992. A random-PCR method (rPCR) to construct whole cDNA library from low amounts of RNA. Nucleic Acids Res. **20:**2900.

12. **Galperin, M. Y., T. A. Gaidenko, A. Y. Mulkidjanian, M. Nakano, and C. W. Price.** 2001. MHYT, a new integral membrane sensor domain. FEMS Microbiol. Lett. **205:**17-23.

13. **Garcia, B., C. Latasa, C. Solano, F. Garcia-del Portillo, C. Gamazo, and I. Lasa.** 2004. Role of the GGDEF protein family in *Salmonella* cellulose biosynthesis and biofilm formation. Mol. Microbiol. **54:**264-277.

14. **Graham, J. E., and J. E. Clark-Curtiss.** 1999. Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). Proc. Natl. Acad. Sci. U S A **96:**11554-11559.

15. **Graham, J. E., R. M. Peek, Jr., U. Krishna, and T. L. Cover.** 2002. Global analysis of *Helicobacter pylori* gene expression in human gastric mucosa. Gastroenterology **123:**1637-1648.

16. **Hirose, K., T. Ezaki, M. Miyake, T. Li, A. Q. Khan, Y. Kawamura, H. Yokoyama, and T. Takami.** 1997. Survival of Vi-capsulated and Vi-deleted *Salmonella typhi* strains in cultured macrophage expressing different levels of CD14 antigen. FEMS Microbiol. Lett. **147:**259-265.

17. **Hook, E. W.** 1985. *Salmonella* species (including typhoid fever). *In* R. G. D. a. J. E. B. e. G.L. Mandell (ed.), *In*: Principles and practices in infectious diseases. Wiley and Sons, NY.

18. **Lostroh, C. P., and C. A. Lee.** 2001. The *Salmonella* pathogenicity island-1 type III secretion system. Microbes Infect. **3:**1281-1291.

19. **Low, D., Braateen, B., Van Der Woude, M.** 2004. Fimbriea. *In* F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology. ASM Press.

20. **Marcus, S. L., J. H. Brumell, C. G. Pfeifer, and B. B. Finlay.** 2000. *Salmonella* pathogenicity islands: big virulence in small packages. Microbes Infect. **2:**145-156.

21. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852-856.

22. **Nair, S., S. Alokam, S. Kothapalli, S. Porwollik, E. Proctor, C. Choy, M. McClelland, S. L. Liu, and K. E. Sanderson.** 2004. *Salmonella enterica* serovar Typhi strains from which SPI7, a 134-kilobase island with genes for Vi exopolysaccharide and other functions, has been deleted. J. Bacteriol. **186:**3214-23.

23. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848-852.

24. **Pickard, D., J. Wain, S. Baker, A. Line, S. Chohan, M. Fookes, A. Barron, P. O. Gaora, J. A. Chabalgoity, N. Thanky, C. Scholes, N. Thomson, M. Quail, J. Parkhill, and G. Dougan.** 2003. Composition, acquisition, and distribution of the Vi exopolysaccharide-encoding *Salmonella enterica* pathogenicity island SPI-7. J. Bacteriol. **185:**5055-5065.

25. **Porwollik, S., E. F. Boyd, C. Choy, P. Cheng, L. Florea, E. Proctor, and M. McClelland.** 2004. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. J. Bacteriol. **186:**5883-5898.

26. **Reed, M. R., K. E. Shearwin, L. M. Pell, and J. B. Egan.** 1997. The dual role of Apl in prophage induction of coliphage 186. Mol. Microbiol. **23:**669-681.

27. **Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush.** 2003. TM4: a free, open-source system for microarray data management and analysis. Biotech. **34:**374-378.

28. **Sharma, A., and A. Qadri.** 2004. Vi polysaccharide of *Salmonella typhi* targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses. Proc. Natl. Acad. Sci. U S A **101:**17492-17497.

29. **Stanley, T. L., C. D. Ellermeier, and J. M. Slauch.** 2000. Tissue-specific gene expression identifies a gene in the lysogenic phage Gifsy-1 that affects *Salmonella enterica* serovar typhimurium survival in Peyer's patches. J. Bacteriol. **182:**4406-4413.

30. **Thomson, N., S. Baker, D. Pickard, M. Fookes, M. Anjum, N. Hamlin, J. Wain, D. House, Z. Bhutta, K. Chan, S. Falkow, J. Parkhill, M. Woodward,**

A. Ivens, and G. Dougan. 2004. The role of prophage-like elements in the diversity of *Salmonella enterica* serovars. J. Mol. Biol. **339:**279-300.

31. **Townsend, S. M., N. E. Kramer, R. Edwards, S. Baker, N. Hamlin, M. Simmonds, K. Stevens, S. Maloy, J. Parkhill, G. Dougan, and A. J. Baumler.** 2001. *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. Infect. Immun. **69:**2894-2901.

32. **Tyler, J. S., M. J. Mills, and D. I. Friedman.** 2004. The operator and early promoter region of the Shiga toxin type 2-encoding bacteriophage 933W and control of toxin expression. J. Bacteriol. **186:**7670-7679.

33. **Wagner, P. L., M. N. Neely, X. Zhang, D. W. Acheson, M. K. Waldor, and D. I. Friedman.** 2001. Role for a phage promoter in Shiga toxin 2 expression from a pathogenic *Escherichia coli* strain. J. Bacteriol. **183:**2081-2085.

34. **Waterman, S. R., and D. W. Holden.** 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cell. Microbiol. **5:**501-511.

35. **Zhou, D., and J. Galan.** 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect. **3:**1293-1298.

FIGURE LEGENDS

Figure 1. Dendogram presenting the hierarchical clustering of *S. typhi*-specific gene expression in different growth conditions as determined by hybridization with SCOTS cDNA probes. Signal intensities were background subtracted and normalized to the

IS*200* control. The log (base 2) of this ratio was used for hierarchical clustering and graphical representation.

Figure 2. Relative expression levels of *S. typhi*-specific genes under different growth conditions (see Fig. 1 for details). A) Genes with signal intensity greater in THP-1 than in RAW264.7. B) Genes with signal intensity equal in both macrophages. C) Genes with signal intensity lower in THP-1 than in RAW264.7.

TABLE 1. Unique ORF identified

Class	ORF	Gene and/or possible function	Locus (size, Kb)	Presence	
				BLAST[a]	Array[b]
SPI-7			STY4521-4680 (134)	Ty	Ty[c], Du[c], Pa[c]
	STY4526	hypothetical protein			
	STY4530	*topB,* topoisomerase B			
	STY4534	hypothetical protein			
	STY4536	*ssb,* single strand binding protein			
	STY4539	*pilL,* putative exported protein			
	STY4573	hypothetical protein			
	STY4574	hypothetical protein			
	STY4589	hypothetical protein			
	STY4643	*apl,* Phage regulatory protein,	prophage $SopE_{ST}$		
	STY4650	*yjhP,* hypothetical protein			
	STY4654	*vexB,* Vi polysaccharide export			
	STY4656	*tviE,* Vi polysaccharide biosynthesis			
	STY4664	putative DNA helicase			
	STY4669	putative membrane protein			
SPI-8	STY3280	bacteriocin fragment	STY3273-3292 (7)	Ty, Pa	Ty, Ag, Pa, Sf
SPI-10	STY4848	putative transposase	STY4821-4853 (33)	Ty, Pa	Ty, Pa, Ja[c],
Prophage					
ST15	STY1635	putative bacteriophage protein	STY1591-1643 (34)	Ty	Ty[c]
	STY1643	*min* DNA-invertase			

ST18	STY2005	conserved hypothetical protein	STY2003-2077 (49.3) ISP1820 : Δ2038-2077	Ty	Ty[c], Cs[c], Pa, Pc[c]
	STY2016	putative bacteriophage protein			
	STY2020	putative bacteriophage protein			
	STY2022	putative exported protein			
	STY2026	putative bacteriophage protein			
ST35	STY3663	hypothetical protein	STY3658-3706 (35)	Ty	Ty, Du[c,c],
	STY3695	*pin,* DNA-invertase			Mo[c,c],
Fimbriae	STY0206	*staB,* chaperone protein	STY0201-0207 (4.7)	Ty	Ty, Se[d], Mo, Or
	STY0207	*staA,* fimbrial protein			
	STY3920	*stgC* , usher	STY3918-3922 (5)	Ty	Ty[d], Se[d], Du[d], En[d]
Regulator	STY3845	*yiiF,* CopG family	STY3844-3845 (0.8)	Ty, Pa	Ty, Pa, Ja
	STY4412	DeoR family	STY4412-4415 (4)	Ty, Pa, En, Du	Ty, Pa, Du, En, Ga, Ja, Me, Mo, Mu, Or,
Other	STY0115	putative IS transposase	STY0114-0115 (1.6)	Ty	Ty, Pa, Ag, Mu
	STY2732	conserved hypothetical protein	STY2731-2732 (2.5)	Ty, Pa, Bo, En, Du	n.a.
	STY3068	hypothetical protein	STY3064-3071 (9)	Ty, Pa	Ty, Pa, Se, Sf
	STY3948	hypothetical protein	STY3948-3950 (3)	Ty, Bo	Ty, Bo, Ag, Se, Sf,
	STY3950	hypothetical protein			
	STY4221	putative aminotransferase	STY4216-4222 (6)	Ty, Pa	Ty, Pa

Ag: *S. agona*, Bo: *S. bongori,* Cs: *S. choleraesuis*, Du: *S. dublin*, En: *S. enteritidis*, Ga: *S. gallinarum*, Ja: *S. java*, Me: *S. muenster*, Mo: *S. montevideo*, Mu: *S. muenchen*, Or: *S. oranienburg*, Pa: *S. paratyphi* A, Pc: *S. paratyphi* C, Se: *S. sendai*, Sf: *S. senftenberg*, Ty: *S. typhi*,

n.a.: not available

[a] Include unfinished genome (see text)

[b] Microarray data from {Porwollik, 2004 #312}. Indicated serovars that presence was in all strains tested from the same serovar.

[c] Microarray data from {Thomson, 2004 #318}

[d] Southern blot hybridization from {Townsend, 2001 #307}

[e] Full-length region not present in all isolates tested

FIGURES

Figure 1.

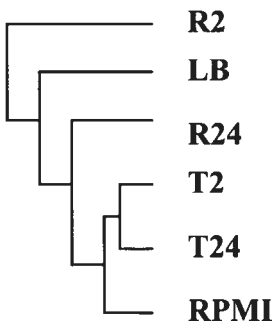
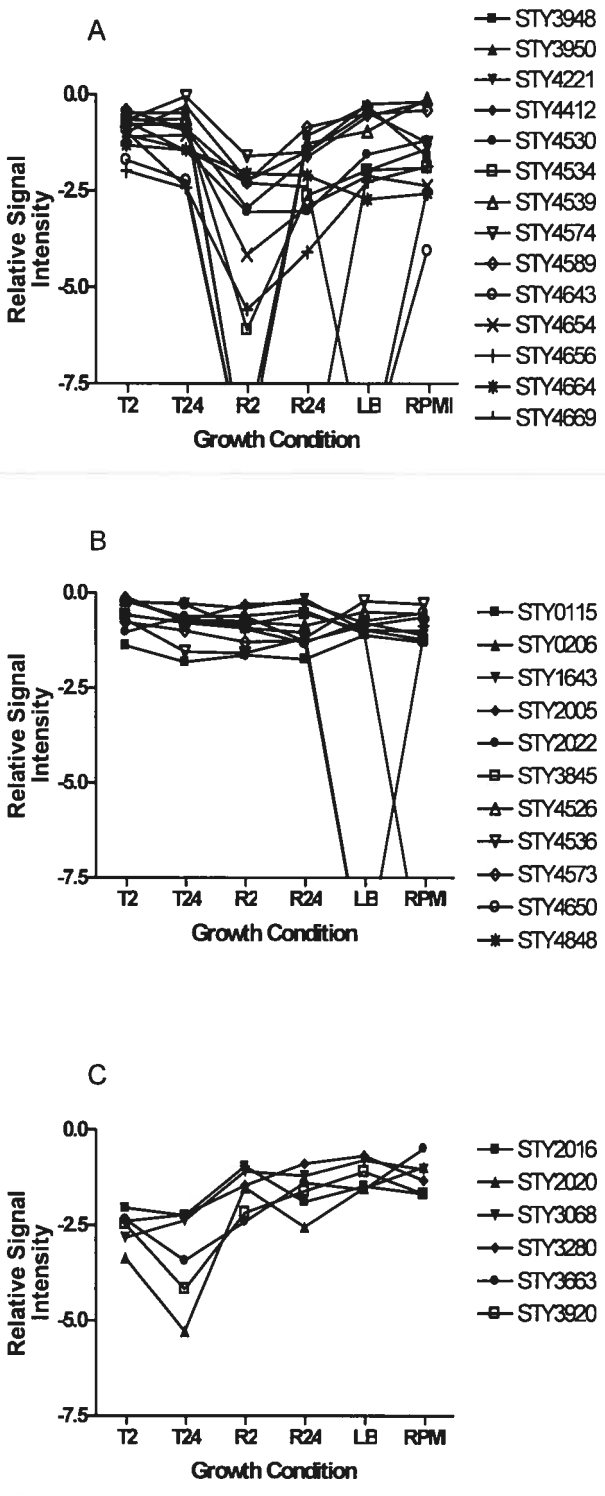


Figure 2


A
Relative Signal Intensity
0.0
-2.5
-5.0
-7.5
T2
T24
R2
R24
LB
RPMI
Growth Condition
STY3948
STY3950
STY4221
STY4412
STY4530
STY4534
STY4539
STY4574
STY4589
STY4643
STY4654
STY4656
STY4664
STY4669
B
Relative Signal Intensity
0.0
-2.5
-5.0
-7.5
T2
T24
R2
R24
LB
RPMI
Growth Condition
STY0115
STY0206
STY1643
STY2005
STY2022
STY3845
STY4526
STY4536
STY4573
STY4650
STY4848
C
Relative Signal Intensity
0.0
-2.5
-5.0
-7.5
T2
T24
R2
R24
LB
RPMI
Growth Condition
STY2016
STY2020
STY3068
STY3280
STY3663
STY3920

Préface au Chapitre 3

La suite logique du premier article est de caractériser certains des gènes identifiés par SCOTS. Le gène *stgC* de l'opéron fimbriaire *stg* a été capturé (Chapitre 2). Il est donc exprimé dans les macrophages et absent dans le génome de Typhimurium. Puisque le rôle des fimbriae spécifiques à Typhi n'a pas été caractérisé pendant l'infection, l'implication de l'opéron fimbriaire *stg* dans la pathogenèse de Typhi a été évaluée. Cet article a été accepté pour publication dans le journal « Infection and Immunity ».

Les expériences décrites dans cet article ont été effectuées par Chantal Forest, moi-même et Katherine Poirier. J'ai aussi supervisé les expériences réalisées par mes collègues. Sébastien Houle, Charles M. Dozois et moi-même sommes responsables de la microscopie électronique. France Daigle a écrit le manuscrit. Tous les coauteurs ont révisé le manuscrit. Les fonds nécessaires à ce projet ont été fournis par France Daigle.

Chapitre 3, 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 Typhi during infection of human macrophages. The *stg* genes are located in the *glmS-pstS* intergenic region in Typhi and certain *E. coli*, but are absent in other *Salmonella enterica* serovars. We cloned the *stg* fimbrial operon into a nonfimbriated *E. coli* K-12 strain and into *Salmonella enterica* serovar Typhimurium. We have demonstrated that the *stg* fimbrial operon contributed to increased adherence to human epithelial cells. Transcriptional fusion assays in serovar Typhi suggest that *stg* is preferentially expressed in minimal medium. The deletion of *stg* reduced adherence of Typhi to epithelial cells. However, the deletion of *stg* increased uptake of Typhi by human macrophages and its overexpression in Typhi and Typhimurium 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 ORF encoding the usher, and is therefore considered a pseudogene, our results show that the *stg* operon may encode for a functional fimbriae. Thus, this Typhi-specific fimbrial operon contributes to interaction with host cells, and further characterization will be important to understand the role of the *stg* fimbrial cluster in typhoid pathogenesis.

INTRODUCTION

The genus *Salmonella* is composed of two species, *Salmonella bongori* and *Salmonella enterica*. *Salmonella enterica* comprises more than 2400 serovars (11) and has been classified into seven subspecies (19). Subspecies I contains serovars Typhi and Typhimurium and most of the serovars that cause diseases in humans and other warm-blooded animals. Some serovars, such as Typhimurium, cause disease in a variety of animals whereas other serovars, such as Typhi, cause disease only in 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 type of disease these serovars elicit in humans, systemic and localized respectively, suggest that one difference between these pathovars might be in the way 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 (22, 29). The Typhi genome of strain CT18 contains 601 genes located on 82 unique genomic regions that are absent from serovar Typhimurium strain LT-2 (29). Thus, it is likely that Typhi possesses unique genetic information that may be important for systemic spreading and survival in the human host. The largest unique region in Typhi is 134 kb and was named *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 polysaccharidic capsule involved in preventing IL-8 production, thus reducing neutrophil influx in the

intestine (31, 33). The *pil* genes encoding for Type IV pili, facilitate bacterial entry into human epithelial cells and are also located on SPI-7 (43).

After ingestion, 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 into the 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 as an essential step by allowing 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 termed *bcf, csg (agf), fim, saf, sef, sta, stb, stc, std, ste, stg, sth* and *tcf*, as well as *pil,* encoding 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 identified *STY3920* (*stgC*), a gene encoding the usher of the

putative *stg* fimbrial operon, as absent in serovar Typhimurium and expressed by serovar Typhi during infection of human macrophages (6). *stgC* contains a premature stop codon that disrupts the predicted ORF encoding the usher, and is therefore considered a pseudogene. As similar fimbrial clusters in *E. coli* also contain genes with premature stop codons and have functional roles (7, 14, 26, 37), we hypothesized that the *stg* operon may encode for a functional fimbriae that contribute to the interaction of Typhi with human cells. In this study, we have 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, except when indicated. When required, antibiotics, amino acids or supplements were added at the following concentrations: kanamycin (Km), ampicillin (Ap), diaminopimelic acid (DAP), 50 μg/ml; chloramphenicol (Cm), 34 μg/ml; tryptophan, cysteine and arginine, 22 μg/ml. Transformation of bacterial strains was routinely done by using the calcium/manganese based (CCMB) or electroporation methods as described (27).

Cloning of the *stg* fimbrial operon. The *stg* operon was amplified from genomic DNA of strain ISP1820 using Elongase® enzyme mix (Invitrogen) with the primers StgA-F

(5'CGGGATCCGAGATGAGAATAACGGAATA-3') containing a *Bam*HI restriction site (underlined) and StgD-R (5'GCTCTAGACATTGATATGACTTATTTTG-3') containing a *Xba*I restriction site (underlined). The 5 kb PCR product was purified and cloned into vector pCR2.1 using the TOPO®XL PCR cloning kit (Invitrogen) resulting in plasmid pSIF018. The *Xba*I and *Hind*III fragment was subcloned into low-copy 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 *Salmonella enterica* serovar Typhimurium and serovar Typhi strains.

Adherence to human epithelial cells. The ability of *E. coli* strain ORN172 containing the *stg* operon (pSIF018) or the vector only (pCR2.1) to adhere to human epithelial cells (INT-407) was assessed. Cells (2.5×10^5) grown in MEM medium (Wysent) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Wysent) and 25 mM HEPES (Wysent), were seeded in 24-well tissue culture plates 24 h before the adherence assays. An hour before infection, cells were washed 3 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 plates and were resuspended in PBS to an OD_{600} of 1.5 ($\approx 1.5 \times 10^9$ CFU). Approximately 2.5×10^7 CFU were added to each well (multiplicity of infection (MOI) of 100). The 24-well plates were then centrifuged at 1000 *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-DOC 0.1% (deoxycholic acid sodium salt) was added to each well, and samples were diluted and spread on LB plates for enumeration by

viable colony counts. Data are 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 bacteria were grown overnight without shaking in LB containing 0.3 M NaCl, and a MOI of 20 was used. When indicated, an additional incubating period of 90 min 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 mix (Invitrogen) and the following primers: StgA-F and StgA-R (5'AACTGCAGCCAGCAAATGCCGTTTTGTT3'). The PCR product was cloned into vector pCR2.1 using the TOPO®XL PCR cloning kit (Invitrogen) resulting in plasmid pSIF016. A 530 bp fragment digested with *Xba*I and *Spe*I was purified and ligated to pFUSE digested with *Xba*I (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 Typhi, pSIF020 was transferred by conjugation and integrated into the genome by homologous recombination as described (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 Typhi strain with a *stg* deletion. A suicide vector for deletion of the *stg* fimbrial operon (STY3918-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 *Xba*I and *Bam*HI 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 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 (16). Isogenic strain DEF004 contains a deletion of the *stg* region resulting from a double crossover, as determined by 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% FCS, 25 mM HEPES, 2 mM L-glutamine, and 1% MEM non-essential amino acids (Wisent) and 1 mM of sodium pyruvate (Sigma). A stock culture of these cells was maintained as monocyte-like, non-adherent 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 (PMA) (Sigma) for 24 to 72 h. For macrophage infection assays, cells were seeded at 5 x 10^5 cells per well in 24-well tissue culture dishes. Bacteria grown overnight at 37°C in static conditions were added to the cell monolayer at a MOI of 10, and centrifuged for 5 min at 1000 *g* to synchronize phagocytosis. After incubation for 20 min at 37°C (T0), the infected cells were washed three times with prewarmed PBS and incubated with supplemented medium as 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% DOC in PBS or further incubated. After lysis the number of surviving bacteria was determined by bacterial plate counts (CFU). Phagocytosis was calculated as a percentage of the initial inoculum. The survival rate corresponds to the percentage of intracellular bacteria at a corresponding time compared to the previous time. Statistical differences were assessed using Student's *t*-test. When 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 addition of cytochalasin D was maintained throughout the infection.

RESULTS

***stg* fimbrial operon.** The *stg* fimbrial cluster possesses a G-C content of 49% and is a member of a distinct group of related fimbriae that are located in the *glmS-pstS* intergenic region (21, 39). The presence of this fimbrial cluster in the sequenced

genomes of *Salmonella enterica* (including unfinished genomes) was only identified 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 previous distribution of *stg* performed by Southern blotting may therefore represent cross-hybridization with other less homologous fimbrial genes (39). However, a putative fimbria inserted in the *glmS-pstS* region in *Salmonella bongori* belongs to the Stg group and possesses the highest identity to the predicted *stg* fimbrial gene products of Typhi (Table 2). A number of fimbrial systems in pathogenic *E. coli* are also inserted in the *glmS-pstS* region and belong to the Stg group, including Stg (21), Lpf_{O113} (5) and Lpf2 (O-island 154) (38). In addition, Lpf and related fimbriae encoded in the *yhjX-yhjW* region in *Salmonella* and *E. coli* (36, 37) share some degree of identity to the predicted *stg* gene products of Typhi but to a lesser extent than other fimbriae belonging to the Stg group (Table 2). The 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 encode for a 170 amino acids (aa) protein and a second ORF designated *stgC'* may encode for a 605 aa protein. The *stgC* stop codon is present in the *stgC* sequence of Typhi strain ISP1820 (data not shown), as well as in the sequenced genomes of 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 non-invasive 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, in between the cells or without pattern on the cell surface and were often found 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 percentage of adhesion to epithelial cells, 3-fold more than the strain harboring the vector alone (Fig. 1C). A higher level of adhesion was also observed when a low copy vector (pSIF026) was used (data not shown).

Adhesion of 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. Typhimurium strain χ3339 harboring *stg* (pSIF018) demonstrated a significantly higher percentage of adhesion to INT-407 cells, thirty-fold more than the strain harboring the vector alone (pCR2.1) (Fig. 1C). As *Salmonella* are able to invade epithelial cells, the level of invasion was also determined by gentamicin protection assay. A similar invasion level to that demonstrated by the wild-type parent harboring the vector only was observed (data not shown).

***stg* expression in Typhi.** To study the expression of the *stg* fimbrial operon in the native Typhi strain, a *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 from early log phase to stationary phase. β-galactosidase expression increased with the growth phase and was highest during stationary phase, following overnight growth in LB (Fig. 2). β-galactosidase expression following growth on LB agar was nearly two-fold higher (54 U) compared to overnight growth in LB broth (29 U) (Fig. 2). The highest expression levels of β-galactosidase 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 (NaCl) concentration of the medium was evaluated, as it 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. These conditions did not result in any significant change in β-galactosidase expression (data not shown).

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

Loss of *stg* results in increased phagocytosis of Typhi by macrophages. As survival in macrophages plays an essential role for 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 h and 24 h post-infection were determined. The mutant showed a significantly higher level of phagocytosis than the wild-type strain (Fig. 3B). Bacterial survival at 2 h or 24 h post-infection was similar for both the *stg* mutant and the wild-type strain (Fig. 3B). Complementation of the *stg* mutant with *stg* on a low-copy vector (pSIF026) restored the wild-type phenotype of phagocytosis (Fig. 3B).

Role of *stg* in macrophage interactions. As bacterial uptake of the *stg* mutant by macrophages was altered, we then wanted to evaluate the effect of *stg* overexpression on phagocytosis. Typhi strain ISP1820 as well as Typhimurium strain χ3339 harboring *stg* (pSIF018) on a multicopy vector both demonstrated a significantly lower level of bacterial uptake than the 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 level 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 than 2% of the initial inoculum was associated with macrophages. The percentage of Typhi that was associated with macrophages was similar when *stg* was present in high-copy number compared to the wild-type harboring the vector alone (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 association with macrophages was similar in cytochalasin D-treated cells regardless of the presence of *stg*, these results indicate that the *stg* fimbrial system contributes to a reduction of internalization of Typhi by macrophages.

DISCUSSION

Bacterial adhesion to host cells is often considered as 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 *Salmonella enterica*. However, only a few systems are 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 difference in binding and host specificities observed between different *Salmonella* serovars. Serovar Typhi is human restricted and harbors 13 putative fimbrial systems and a Type IV pilus (29). We have previously identified that *stg* is transcribed by serovar Typhi within macrophages (6). In *Salmonella enterica*, the *stg* fimbrial cluster located in the *glmS-pstS* region is only present in serovar Typhi (30).

The *stg* gene cluster was suggested to be non-functional 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 fimbria from the bacterial surface (18). The fimbrial usher protein family consists of a group of large proteins (800–900 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 fimbriae encoding *lpf1* 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 and determine if the *stg* 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, it is possible that other fimbrial ushers present in the cell may function for Stg, or 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 detected these fimbriae using wild-type strains, and fimbrial proteins or structures were only detected 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 blot using an anti-StgA from *E. coli* (21). One explanation for a lack of fimbrial structures despite an adhesion phenotype may be that some export and partial assembly of Stg protein occurs, which results in an adhesin that is not filamentous. Stg and related fimbriae demonstrate 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 a *stgA-lacZ* single copy fusion, a low level of *stg* expression was also detected in different growth conditions. The highest levels of *stg* expression were obtained when bacteria were grown in minimal medium or on solid medium (Fig. 2), and were not influenced by the presence of salts or iron. The low level of fimbrial gene expression observed during in vitro growth of Typhi is similar to results obtained with Typhimurium (13). In Typhimurium, which contains 13 fimbrial operons (22), only Type 1 fimbriae were expressed in vitro at 37 °C. 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 unknown why expression of many fimbrial systems is suppressed in vitro.

While being 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 the expression of fimbrial antigens during the 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 *Salmonella enterica* serovar Typhimurium, from 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 Typhi determinants identified so far that confer adherence to human epithelial cells (43). Redundancy of virulence determinants is not uncommon. Wild-type virulent Typhi strains lacking SPI-7, which harbors Type IV pili, have been isolated (25), suggesting that *stg* fimbrial operon may confer adhesion to host cells in *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 adherence of 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 Typhi as well as in Typhimurium (Fig. 4). The higher level of phagocytosis in the absence of the *stg* genes may have been caused by an 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 polymerisation which mediates uptake of bacteria. The number of bacteria associated cytochalasin D-treated with macrophages was similar for the wild-type, the *stg* mutant strain as well as 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 is not due to a decrease of association with macrophages, but likely to a specific reduction of phagocytic activity. By contrast, Type IV pili increased entry of 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 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 primarily be involved in initial contact with host cells. It is possible that the *stg* fimbrial operon may promote inhibition of

phagocytosis to evade inflammatory cells of the intestine in order to be able to invade deeper tissue.

The data presented in this paper demonstrate that the *stg* gene cluster of Typhi expresses a functional and serovar specific-adhesin. The *stg* gene cluster potentially contribute to the initial stage of typhoid pathogenesis by mediating adherence of Typhi to host epithelial cells and by inhibiting phagocytosis. It will be 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.

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REFERENCES

1. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703-714.

2. **Baumler, A. J., R. M. Tsolis, A. W. van der Velden, I. Stojiljkovic, S. Anic, and F. Heffron.** 1996. Identification of a new iron regulated locus of *Salmonella typhi*. Gene **183:**207-213.

3. **Daigle, F., J. E. Graham, and R. Curtiss, 3rd.** 2001. Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). Mol. Microbiol. **41:**1211-1222.

4. **Deng, W., S. R. Liou, G. Plunkett, 3rd, G. F. Mayhew, D. J. Rose, V. Burland, V. Kodoyianni, D. C. Schwartz, and F. R. Blattner.** 2003. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. J. Bacteriol. **185:**2330-2337.

5. **Doughty, S., J. Sloan, V. Bennett-Wood, M. Robertson, R. M. Robins-Browne, and E. L. Hartland.** 2002. Identification of a novel fimbrial gene cluster related to long polar fimbriae in locus of enterocyte effacement-negative strains of enterohemorrhagic *Escherichia coli*. Infect. Immun. **70:**6761-6769.

6. **Faucher, S. P., R. Curtiss, 3rd, and F. Daigle.** 2005. Selective capture of *Salmonella enterica* serovar Typhi genes expressed in macrophages that are absent from the *Salmonella enterica* serovar Typhimurium genome. Infect. Immun. **73:**5217-5221.

7. **Fitzhenry, R., S. Dahan, A. G. Torres, Y. Chong, R. Heuschkel, S. H. Murch, M. Thomson, J. B. Kaper, G. Frankel, and A. D. Phillips.** 2006. Long polar fimbriae and tissue tropism in *Escherichia coli* O157:H7. Microbes Infect. **8:**1741-1749.

8. **Galan, J. E., and R. Curtiss III.** 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. **58:**1879-1885.

9. **Gulig, P. A., and R. I. Curtiss.** 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. **55:**2891-2901.

10. **Harris, J. B., A. Baresch-Bernal, S. M. Rollins, A. Alam, R. C. LaRocque, M. Bikowski, A. F. Peppercorn, M. Handfield, J. D. Hillman, F. Qadri, S. B. Calderwood, E. Hohmann, R. F. Breiman, W. A. Brooks, and E. T. Ryan.** 2006. Identification of in vivo-induced bacterial protein antigens during human infection with *Salmonella enterica* serovar Typhi. Infect. Immun. **74:**5161-5168.

11. **Hook, E. W.** 1985. *Salmonella* species (including typhoid fever), p. 1256-1269. *In* G. L. Mandell, R. G. Douglas, and J. E. Bennett (ed.), Principles and practices in infectious diseases. Wiley and Sons, NY.

12. **Humphries, A., S. Deridder, and A. J. Baumler.** 2005. *Salmonella enterica* serotype Typhimurium fimbrial proteins serve as antigens during infection of mice. Infect. Immun. **73:**5329-5338.

13. **Humphries, A. D., M. Raffatellu, S. Winter, E. H. Weening, R. A. Kingsley, R. Droleskey, S. Zhang, J. Figueiredo, S. Khare, J. Nunes, L. G. Adams, R. M. Tsolis, and A. J. Baumler.** 2003. The use of flow cytometry to detect

expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. Mol. Microbiol. **48:**1357-1376.

14. **Jordan, D. M., N. Cornick, A. G. Torres, E. A. Dean-Nystrom, J. B. Kaper, and H. W. Moon.** 2004. Long polar fimbriae contribute to colonization by *Escherichia coli* O157:H7 in vivo. Infect. Immun. **72:**6168-6171.

15. **Kaniga, K., M. S. Compton, R. Curtiss, 3rd, and P. Sundaram.** 1998. Molecular and functional characterization of *Salmonella enterica* serovar Typhimurium *poxA* gene: effect on attenuation of virulence and protection. Infect. Immun. **66:**5599-5606.

16. **Kaniga, K., I. Delor, and G. Cornelis.** 1991. A wide host range suicide vector for improving reverse genetics in gram negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137-141.

17. **Klugman, K. P., I. T. Gilbertson, H. J. Koornhof, J. B. Robbins, R. Schneerson, D. Schulz, M. Cadoz, and J. Armand.** 1987. Protective activity of Vi capsular polysaccharide vaccine against typhoid fever. Lancet **2:**1165-1169.

18. **Kuehn, M. J., F. Jacob-Dubuisson, K. Dodson, L. Slonim, R. Striker, and S. J. Hultgren.** 1994. Genetic, biochemical, and structural studies of biogenesis of adhesive pili in bacteria. Methods Enzymol. **236:**282-306.

19. **LeMinor, L., and M. Y. Popoff.** 1987. Designation of *Salmonella enterica* sp. nov., nom. rev., as the type and only species of the genus *Salmonella*. Int. J. Syst. Bacteriol. **37:**465-468.

20. **Low, A. S., F. Dziva, A. G. Torres, J. L. Martinez, T. Rosser, S. Naylor, K. Spears, N. Holden, A. Mahajan, J. Findlay, J. Sales, D. G. Smith, J. C. Low, M. P. Stevens, and D. L. Gally.** 2006. Cloning, expression, and characterization

of fimbrial operon F9 from enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **74:**2233-2244.

21. **Lymberopoulos, M. H., S. Houle, F. Daigle, S. Leveille, A. Bree, M. Moulin-Schouleur, J. R. Johnson, and C. M. Dozois.** 2006. Characterization of Stg fimbriae from an avian pathogenic *Escherichia coli* O78:K80 strain and assessment of their contribution to colonization of the chicken respiratory tract. J. Bacteriol. **188:**6449-6459.

22. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852-856.

23. **Miller, J. H.** 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, NY, USA.

24. **Mol, O., and B. Oudega.** 1996. Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli*. FEMS Microbiol. Rev. **19:**25-52.

25. **Nair, S., S. Alokam, S. Kothapalli, S. Porwollik, E. Proctor, C. Choy, M. McClelland, S. L. Liu, and K. E. Sanderson.** 2004. *Salmonella enterica* serovar Typhi strains from which SPI7, a 134-kilobase island with genes for Vi exopolysaccharide and other functions, has been deleted. J. Bacteriol. **186:**3214-3223.

26. **Newton, H. J., J. Sloan, V. Bennett-Wood, L. M. Adams, R. M. Robins-Browne, and E. L. Hartland.** 2004. Contribution of long polar fimbriae to the

virulence of rabbit-specific enteropathogenic *Escherichia coli*. Infect. Immun. **72:**1230-1239.

27. **O'Callaghan, D., and A. Charbit.** 1990. High efficiency transformation of *Salmonella typhimurium* and *Salmonella typhi* by electroporation. Mol. Gen. Genet. **223:**156-158.

28. **Pan, Q., X. L. Zhang, H. Y. Wu, P. W. He, F. Wang, M. S. Zhang, J. M. Hu, B. Xia, and J. Wu.** 2005. Aptamers that preferentially bind type IVB pili and inhibit human monocytic-cell invasion by *Salmonella enterica* serovar Typhi. Antimicrobial agents and chemotherapy **49:**4052-4060.

29. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848-852.

30. **Porwollik, S., E. F. Boyd, C. Choy, P. Cheng, L. Florea, E. Proctor, and M. McClelland.** 2004. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. J. Bacteriol. **186:**5883-5898.

31. **Raffatellu, M., D. Chessa, R. P. Wilson, R. Dusold, S. Rubino, and A. J. Baumler.** 2005. The Vi capsular antigen of *Salmonella enterica* serotype Typhi

reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. Infect. Immun. **73:**3367-3374.

32. **Rosenshine, I., S. Ruschkowski, and B. B. Finlay.** 1994. Inhibitors of cytoskeletal function and signal transduction to study bacterial invasion. Methods Enzymol. **236:**467-476.

33. **Sharma, A., and A. Qadri.** 2004. Vi polysaccharide of *Salmonella typhi* targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses. Proc. Natl. Acad. Sci. U. S. A. **101:**17492-17497.

34. **Smyth, C. J., M. B. Marron, J. M. Twohig, and S. G. Smith.** 1996. Fimbrial adhesins: similarities and variations in structure and biogenesis. FEMS Immunol. Med. Microbiol. **16:**127-139.

35. **Tatsuno, I., R. Mundy, G. Frankel, Y. Chong, A. D. Phillips, A. G. Torres, and J. B. Kaper.** 2006. The *lpf* gene cluster for long polar fimbriae is not involved in adherence of enteropathogenic *Escherichia coli* or virulence of *Citrobacter rodentium*. Infect. Immun. **74:**265-272.

36. **Toma, C., N. Higa, S. Iyoda, M. Rivas, and M. Iwanaga.** 2006. The long polar fimbriae genes identified in Shiga toxin-producing *Escherichia coli* are present in other diarrheagenic *E. coli* and in the standard *E. coli* collection of reference (ECOR) strains. Res. Microbiol. **157:**153-161.

37. **Torres, A. G., J. A. Giron, N. T. Perna, V. Burland, F. R. Blattner, F. Avelino-Flores, and J. B. Kaper.** 2002. Identification and characterization of *lpfABCC'DE*, a fimbrial operon of enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **70:**5416-5427.

38. **Torres, A. G., K. J. Kanack, C. B. Tutt, V. Popov, and J. B. Kaper.** 2004. Characterization of the second long polar (LP) fimbriae of *Escherichia coli* O157:H7 and distribution of LP fimbriae in other pathogenic *E. coli* strains. FEMS Microbiol. Lett. **238:**333-344.

39. **Townsend, S. M., N. E. Kramer, R. Edwards, S. Baker, N. Hamlin, M. Simmonds, K. Stevens, S. Maloy, J. Parkhill, G. Dougan, and A. J. Baumler.** 2001. *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. Infect. Immun. **69:**2894-2901.

40. **Van Rosmalen, M., and M. H. Saier, Jr.** 1993. Structural and evolutionary relationships between two families of bacterial extracytoplasmic chaperone proteins which function cooperatively in fimbrial assembly. Res. Microbiol. **144:**507-527.

41. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-199.

42. **Woodall, L. D., P. W. Russell, S. L. Harris, and P. E. Orndorff.** 1993. Rapid, synchronous, and stable induction of type 1 piliation in *Escherichia coli* by using a chromosomal *lacUV5* promoter. J. Bacteriol. **175:**2770-2778.

43. **Zhang, X. L., I. S. Tsui, C. M. Yip, A. W. Fung, D. K. Wong, X. Dai, Y. Yang, J. Hackett, and C. Morris.** 2000. *Salmonella enterica* serovar Typhi uses type IVB pili to enter human intestinal epithelial cells. Infect. Immun. **68:**3067-3073.

FIGURE LEGENDS

Figure 1. Adherence and expression of the *stg* fimbrial operon by *E. coli* and *Salmonella enterica* serovar Typhimurium. 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. Bacteria are indicated by arrows. The percentage of the initial inoculum associated with epithelial cells after 90 min of incubation is indicated for *E. coli* and serovar Typhimurium carrying the *stg* operon (DEF047) or the vector control (DEF048) (C). All assays were conducted in duplicate and repeated independently at least three times. Results are expressed as the mean ± standard error of the replicate experiments. An asterisk indicates that there is a significant difference between the strain containing the control vector or the *stg* operon ($P < 0.005$).

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

Figure 3. Role of *stg* for interaction of Typhi with human cells. The capacity of the wild-type strain, the *stg* mutant (DEF004), or the complemented strain (DEF066) to adhere 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. Results are expressed as the mean ± standard error of the replicate experiments. Significant differences ($P < 0.005$) in adherence or phagocytosis between the mutant and the wild-type strain of Typhi are indicated by asterisks. The values for percent recovery were normalized to the wild-type control, which was designated 100% at each time point.

Figure 4. Effect of overexpression of *stg* on phagocytosis. Serovar Typhimurium carrying the *stg* cluster (DEF047) or the vector control (DEF048) and serovar Typhi carrying the *stg* cluster (DEF033) or the vector control (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. Results are expressed as the mean ± standard error of the replicate experiments. An asterisk indicates a significant difference in phagocytosis between the wild-type strains containing the vector alone or encoding 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 number of bacteria associated with macrophages with *stg* (DEF033) or without *stg* (DEF004) 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, which was designated 100% at each time point. Results are expressed as the mean ± standard error of the replicate experiments.

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

Strain or plasmid	Characteristics	Reference or source
Salmonella enterica		
Typhi strains		
ISP1820	Wild-type *S.* Typhi	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
Typhimurium strains		
χ3339	Mouse-passaged isolate of SL1344 *rpsL hisG*	(9)
DEF047	χ3339 (pSIF018)	This study
DEF048	χ3339 (pCR2.1)	This study
E. coli		
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-1 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 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' and *stgD* in 3' 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* Typhi with other fimbrial systems [a]

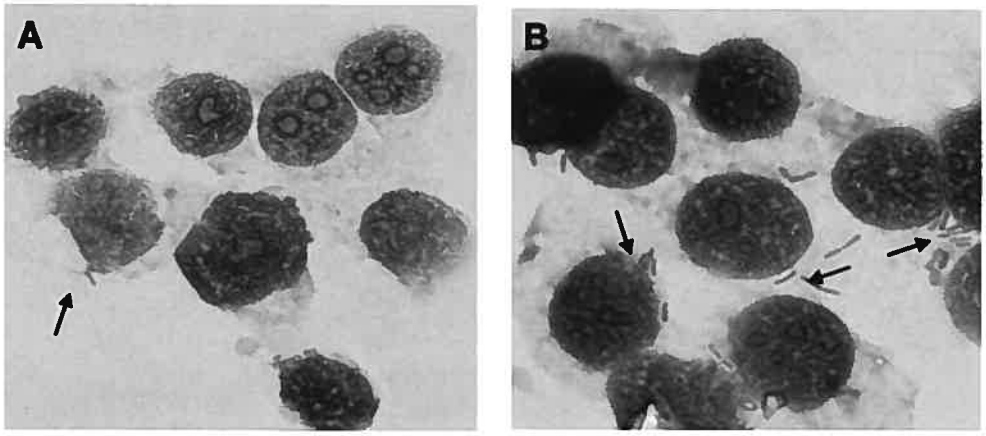
Fimbrial group	Strain	Localization	% Identity (% similarity)			
			StgA	StgB	StgC [b]	StgD
Stg group	*S. bongori*	*glmS-pstS*	70.7 (81.2)	63.7 (73,7)	82.2 (89.8)	38.7 (53.6)
	APEC 078 (*stg*)	*glmS-pstS*	66.5 (79.1)	54.8 (69.8)	67.1 (82.0)	36.6 (54.1)
	EHEC O157 (*lpf2*)	*glmS-pstS*	59.4 (72.8)	62.1 [b] (76.3)	73.5 (85.6)	35.4 (50.3)
	EHEC O113 (lpf_{O113})	*glmS-pstS*	66.5 (79.1)	53.6 (69.4)	67.1 (82.0)	24.7 (37.9)
Lpf group	*S.* Typhimurium LT2	*yhjX-yhjW*	32.5 (45.7)	32.1 (53.3)	40.8 (59.2)	27.9 (45.6)
	EHEC O157 (*lpf1*)	*yhjX-yhjW*	30.5 (44.2)	33.9 (54.8)	38.8 [b] (56.6)	28.0 (44.6)
	REPEC 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

FIGURES

Figure 1



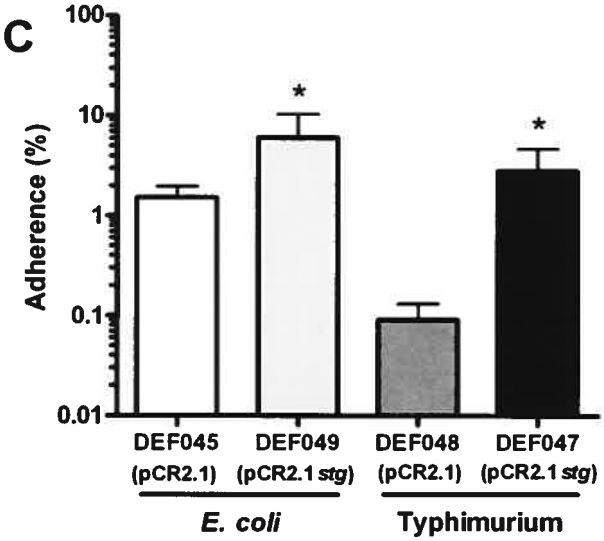


Figure 2

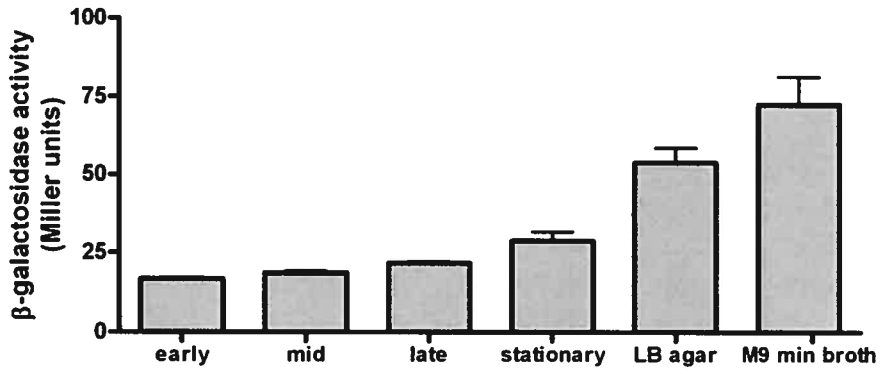


Figure 3

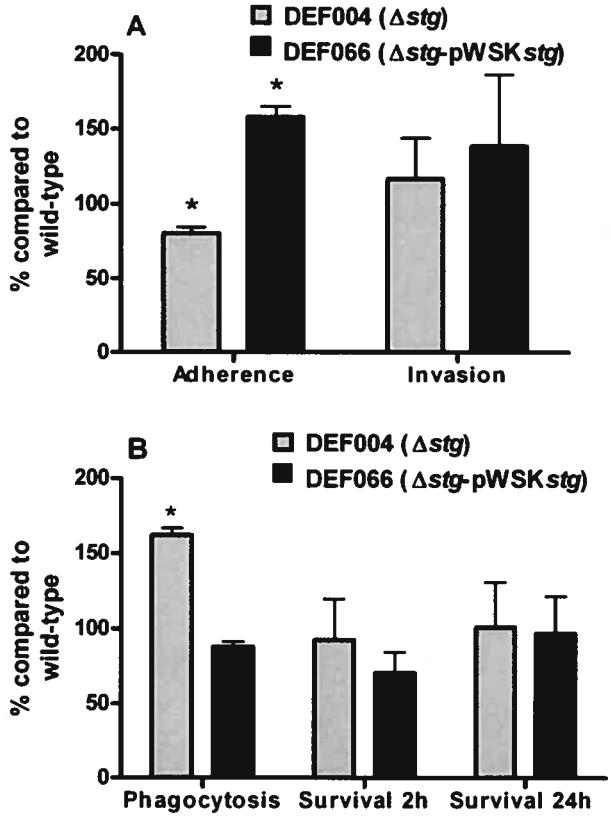


Figure 4

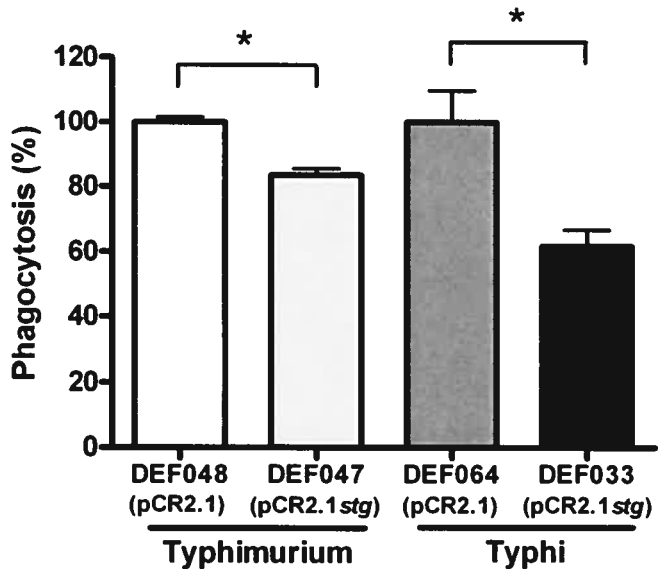
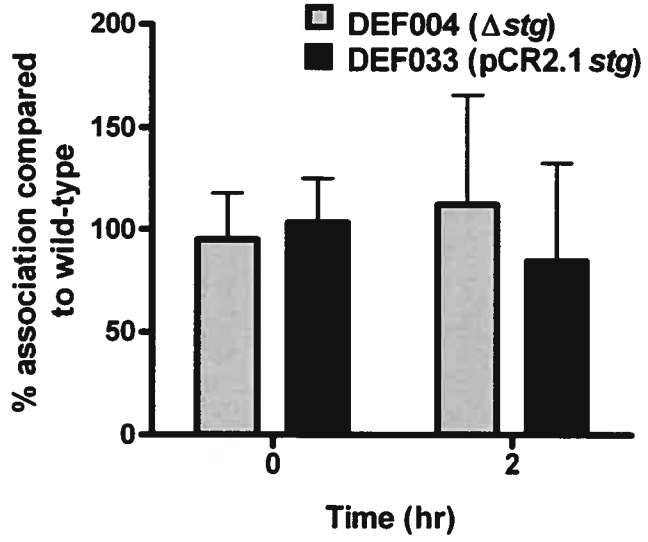


Figure 5



Préface au Chapitre 4

L'approche utilisée au chapitre 2, SCOTS, ne permet pas d'identifier globalement les gènes uniques de Typhi exprimés pendant l'infection des macrophages. La seule approche qui permet d'identifier globalement les gènes induits dans une condition particulière est la méthode des biopuces, comme expliqué dans la section 1.6.4. Par contre, cette méthode est peu appliquée pour étudier des conditions d'infections puisque la quantité d'ARN disponible dans ces conditions est trop faible et mélangée à l'ARN eucaryote. SCOTS est capable de purifier et d'amplifier spécifiquement les transcrits bactériens, à partir de condition d'infection. Il semblait donc possible que l'utilisation de SCOTS pour purifier et amplifier les transcrits bactériens provenant de l'infection puisse permettre l'utilisation des biopuces. Le chapitre 4 décrit l'application de SCOTS en conjonction avec les biopuces pour obtenir le transcriptome de Typhi lors de l'infection de macrophages humains. Le matériel supplémentaire (supporting figures 4-8 and supporting table 1-5) est disponible sur le site web du journal PNAS à l'adresse suivante : http://www.pnas.org/cgi/content/full/0509183103/DC1.

Pour ce manuscrit, j'ai effectué toutes les expériences et l'analyse des résultats obtenus. Steffen Porwollik et Michael McClelland ont imprimé les biopuces et fourni les outils informatiques nécessaires à l'analyse des résultats. Charles M. Dozois a fourni les équipements nécessaires aux expériences de PCR en temps réel. Ce manuscrit a été écrit par moi et France Daigle et a été révisé par tous les coauteurs. Les fonds nécessaires à la réalisation de cette étude ont été fournis par France Daigle et Michael McClelland.

CHAPITRE 4, 3e article

Classification: Biological Sciences, Microbiology

Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences

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Abbreviations: SCOTS, selective capture of transcribed sequences; SPI, *Salmonella* pathogenicity island; TTSS, type three secretion system.

Data deposition: GEO Series accession number GSE3094, GSE3095 and GSE3096.

Running title: Typhi transcriptome within human macrophages

ABSTRACT

The cDNA obtained by SCOTS (Selective Capture Of Transcribed Sequences) is a complex mixture that can be used in conjunction with microarrays to determine global gene expression by a pathogen during infection. We used this method to study genes expressed by *Salmonella enterica* serovar Typhi, the etiological agent of typhoid fever, within human macrophages. Global expression profiles of Typhi grown in vitro and within macrophages at different time points were obtained and compared. Known virulence factors, such as the SPI-1 and SPI-2 encoded type III secretion systems, were found to be expressed as predicted, during infection by *Salmonella*, which validate our data. Typhi inside macrophages showed increased expression of genes encoding resistance to antimicrobial peptides, used the glyoxylate bypass for fatty acid utilization, and did not induce the SOS response or the oxidative stress response. Genes coding for the flagellar apparatus, chemotaxis and iron transport systems were down-regulated in vivo. Many cDNAs corresponding to genes with unknown functions were up-regulated inside human macrophages and will be important to consider for future studies to elucidate the intracellular lifestyle of this human specific pathogen. Real-Time qPCR was consistent with the microarray results. The combined use of SCOTS and microarrays is an effective way to determine the bacterial transcriptome in vivo and could be used to investigate transcriptional profiles of other bacterial pathogens, without the need to recover many nanograms of bacterial mRNA from host, and without increasing the multiplicity of infection beyond what is seen in nature.

INTRODUCTION

Salmonella infections are a significant cause of morbidity in humans and animals. *Salmonella enterica* serovar Typhi (hereafter referred to as Typhi) is the etiological agent of typhoid fever, a major health problem in developing countries, and causes an estimated 16 million cases and 600 000 deaths annually worldwide (1). Publications on human typhoid that use modern immunological and molecular techniques are scarce. We have a very limited knowledge of the pathogenesis of Typhi because Typhi infects only humans, resulting in a lack of virulence assays. Many studies have relied on a murine model of human typhoid that uses *Salmonella enterica* serovar Typhimurium (hereafter referred to as Typhimurium), which causes a typhoid-like disease in mice. Consequently, what is known about Typhi pathogenicity has been largely extrapolated from studies of Typhimurium infections in mice.

The *Salmonella* chromosome possesses insertions of large regions of DNA, containing virulence genes. These *Salmonella* pathogenicity islands (SPIs) play a key role in *Salmonella* pathogenesis (2). Thus far, ten SPIs have been identified in Typhi (3). SPI-1 and SPI-2, which are present in all *S. enterica* serovars, represent two major pathogenesis determinants that encode type III secretion systems (TTSS). SPI-1 and SPI-2 TTSS have distinct roles in *Salmonella* pathogenesis. SPI-1 effectors are injected into host cells via the TTSS and are required for invasion of epithelial cells (4), whereas SPI-2 contributes to *Salmonella* survival inside macrophages (5, 6). The ability to survive and replicate within macrophages is thought to be one of the major pathogenesis determinants for *Salmonella* (7, 8). Following entry into macrophages, *Salmonella*

adapts by modifying its gene expression to respond to the host cell environment. It was recently shown that Typhimurium alters the transcription of 20% of its genome (919 genes) once inside murine macrophages (9). It was previously shown that Typhimurium survives better in murine macrophages than in human macrophages, whereas Typhi survives better in human macrophages than in murine macrophages (10). It is possible that Typhi expresses specific factors for survival in human macrophages. Genomic comparison between Typhi strain CT18 and Typhimurium strain LT2 revealed that there are 601 genes unique to Typhi and 479 genes unique to Typhimurium (3, 11).

Microarray analysis is a powerful tool to expand our current knowledge of global bacterial gene expression, but there are very few studies on bacterial transcriptional response during infection. There are many factors that prevent using DNA microarrays effectively for studying bacterial gene expression in infected host cells or tissues, such as the low amount of bacterial RNA in vivo, the short half-life of bacterial mRNA, and the contamination of bacterial mRNA with rRNA and host RNA (12). To overcome these problems, researchers have used different approaches than can create additional problems, such as models of infection that do not necessarily represent conditions found within host cells or tissues during the infectious process, and which may cause artifactual bacterial gene expression.

Selective Capture of Transcribed Sequences (SCOTS) has been used to identify genes expressed in vivo by bacterial pathogens such as *Mycobacterium* (13, 14), *Salmonella* (15-17), *Actinobacillus* (18), *Helicobacter pylori* (19) and avian pathogenic *Escherichia coli* (20). In this report, we demonstrate that SCOTS can be used to obtain

high-quality transcript profiles from intracellular bacteria such as Typhi within human macrophages, and that the use of microarrays and SCOTS-cDNA hybridization provides an effective means to elucidate the global bacterial expression profile from infected host cells.

RESULTS

SCOTS-cDNA. To verify if SCOTS-derived cDNAs could be successfully used for microarray analysis, we hybridized each of the 3 rounds of SCOTS as well as the cDNA obtained from infected macrophages 2 h post-infection (T2) to *Salmonella* arrays. The initial cDNA obtained from infected macrophages very weakly hybridized to the *Salmonella* microarrays and only a few genes were detected (Fig. 1). This demonstrates the inadequate sensitivity of labeled cDNA and illustrates limitations of direct identification of bacterial gene transcripts during interaction with host cells. The low number of genes detected in the initial cDNA and the low intensities of spots also confirmed the absence of contaminating bacterial genomic DNA, which was confirmed by PCR (data not shown), and the lack of host cDNA cross-hybridization. The numbers of detected genes increased in successive SCOTS rounds (Fig. 1C). This increasing complexity of transcripts was previously visualized by Southern blot (13). All cDNAs used for hybridization to microarrays were obtained by 3 rounds of SCOTS. Nearly 4000 genes were detected using SCOTS-cDNA in each of the conditions, without regard to whether they are up-regulated or down-regulated relative to other growth conditions (see figure 1 legend for detection threshold). This illustrates that the diversity of capture efficiencies were similar for the different conditions tested and that detection was largely

independent of the quantity of bacteria recovered from samples (Fig. S1). Similarly, detection of *Mycobacterium tuberculosis* transcripts using microarrays identified 75% of genes that had measurable signal intensities (21).

The genomic content of Typhi strain ISP1820 was compared to strain CT18, the template used for the DNA array design. Seventy-six CT18 genes were missing or altered in the ISP1820 genome (Fig. S2) and four regions (II, V, IX, and XIII) encoding prophage-like elements, were not detected in strain ISP1820, as previously observed in other Typhi strains (22).

In vivo global transcription profiles. cDNA of Typhi present in the supernatants of infected macrophages was obtained by SCOTS and used as an in vitro control. Typhi cDNA was also obtained by SCOTS from infected macrophages after phagocytosis (T0), and intracellularly at 2 h (T2), 8 h (T8) and 24 h (T24) post-infection. The gene expression profiles for intracellular Typhi were compared with the transcriptome of bacteria from the cell supernatants (Table S1). During the time course of infection, 36% of the Typhi genome showed significant expression differences within macrophages compared to extracellular Typhi obtained from the cell supernatant. The number of Typhi genes down-regulated (2-fold) during infection, compared to bacteria from the cell supernatant, was 490, 474, 705 and 478 genes for T0, T2, T8 and T24 respectively. There were 1129 genes down-regulated by intracellular Typhi from at least one time point when compared to the supernatant (Table S2), and 138 genes were repressed at all intracellular time points (Fig. S3A).

Typhi gene transcripts corresponding to approximately 300 different ORFs were more abundant (2-fold) at each time point (273, 309, 301 and 309 genes at T0, T2, T8 and T24 respectively). A total of 628 genes were up-regulated by intracellular Typhi at one or more time points (Table S3). Some genes were more highly expressed only at one time point and others were up-regulated at two or more time points, and 117 Typhi genes were up-regulated at all intracellular time points (Fig. S3B). These intracellular expressed genes include SPI-2 genes (see below) and other genes involved in virulence such as, *pagC, pagD*, and *mgtBC* and genes encoding osmotically induced proteins (*osmC* and *osmE*) and phage shock protein (*psp*).

SPIs expression. SPI-1 encodes a TTSS required for invasion of non-phagocytic cells (4). We found that some SPI-1 genes were up-regulated during bacterial uptake by macrophages at T0, such as *prg*, *sip*, *spa* and *inv* genes, and then down-regulated after internalization, and for the rest of the infection (Fig. 2A). SPI-2 encodes a second TTSS that is required for *Salmonella* survival inside macrophages (5, 6). In our model, some SPI-2 genes were up-regulated following bacterial uptake by macrophages (T0) and most SPI-2 genes were up-regulated after 2 h infection (Fig. 2B). Our results are consistent with the biological roles of both TTSS. Moreover, it is notable that genes encoding for SPI-1 effectors, such as *sopE*, *sopE2*, *sopB(sigD)* and *sopD*, or SPI-2 effectors such as *pipB* and *sifB*, demonstrated an expression profile similar to their respective TTSS even if encoded outside the SPIs. SPI-3-localized *mgtBC* genes, which encode a Mg^{2+} transporter required for full virulence of Typhimurium in mice (23), were found to be induced in macrophages. No significant difference in expression was observed for SPI-4 and SPI-9 components. The SPI-5 encoded gene *pipD* was over-

expressed within macrophages at all time points. SPI-6 possesses a mixture of genes that were either up-regulated or repressed in vivo. A distinct feature of Typhi compared to Typhimurium is SPI-7, a 134 kb region (24) that encodes the Type-IV pili involved in entry into intestinal cells (25) and the *viaB* locus responsible for the synthesis of the Vi-capsule, involved in the suppression of early inflammatory responses of intestinal cells (26). For SPI-7, 19 genes were up-regulated within macrophages, including *sopE,* a SPI-1 effector, and 47 genes were down-regulated within macrophages for at least one time point, including genes involved in Type-IV pili synthesis and genes involved in Vi capsule biosynthesis. Their role may be more important at the intestinal level than within macrophages. Most of the SPI-8 and SPI-10 encoded genes displayed signals below the background threshold.

Functional classes. Typhi genes that were differentially expressed within macrophages were grouped by their function or genetic locus according to the Typhi functional classification scheme used by the Sanger Centre (http://www.sanger.ac.uk) and the percentage of genes that were up-regulated or down-regulated within macrophages in each of these groups was calculated (Fig. S4). Genes involved in iron acquisition and transport (such as *fes, fhu, feo, ent*) were down-regulated intracellularly (Fig. 2C). Genes involved in chemotaxis and motility, like flagella, were up-regulated during bacterial uptake by macrophages (T0) and then were reduced once bacteria were within macrophages (Fig. 2D). Moreover, the majority of genes involved in response to antimicrobial peptides, including *phoP*, *pmrF*, *ugtL*, *pqaB*, *pgtE*, *mig-14* and *somA* (*ybjX*) were significantly up-regulated in macrophages (Fig. 2E). In addition, the *pagP, virK* and *pmrD* genes, were also more expressed within macrophages than within the

supernatant. From a list of 92 genes induced after treatment of Typhi with peroxide (27), only 18 were significantly up-regulated in vivo, including *phoH* and *mgtC* (Fig. 2F). The *sodB* gene was up-regulated at 2 h and 24 h post-infection, however expression of the genes encoding OxyR, the oxidative stress regulator as well as *katEG* and *sodA* genes were not up-regulated by Typhi in macrophages. Response of Typhi to acid was investigated by looking at a list of genes known to be acid inducible (28, 29) or associated with the acid tolerance response (ATR) (30). No clear differential expression can be seen. However, regulators involved in ATR response such as *rpoS* and *phoP* were induced but are also involved in other functions.

Salmonella has many transcriptional regulators that control virulence gene expression (31), but significant changes in expression levels were observed only for a subset of regulatory genes in Typhi. *phoP* and the *ssrBA* genes, which encode a two-component regulator of SPI-2 gene expression, were strongly up-regulated from T0 to T24. A 2-fold increase was observed for regulatory genes such as *rpoS*, *rpoH*, *fur*, *soxS*, *slyA*, and *phoB* during infection. The expression of other regulators such as *rpoE*, *rpoD*, *hilA*, *barA/sirA*, *oxyR*, *cyaA* and *crp* was not significantly changed.

qPCR analysis. The results obtained with microarray experiments were confirmed by using quantitative real-time PCR (qPCR). qPCR results were consistent with the microarray results (Fig. 3). As an example, both methods showed an increased expression of the SPI-2 gene *sseB* and a decreased expression of the SPI-1 gene *prgI* during infection. Normalization against 16s rRNA gave similar results (data not shown).

DISCUSSION

This report investigated an approach to circumvent many of the technical problems encountered when studying bacterial gene transcription in vivo. We have demonstrated that SCOTS-cDNA represents a complex mixture that can be used for microarray analysis and allowed the study at different times post infection, not only those with relatively large numbers of bacteria, as required by other techniques (12). The transcriptomes of Typhi from macrophage cell culture supernatant (in vitro) or following macrophage uptake and survival at different times post-infection were obtained and compared. Identification of Typhi gene transcription profiles within macrophages may elucidate the role of some Typhi genes, as adaptation and survival within macrophages was shown to be critical (8).

We determined that approximately 300 Typhi genes were up-regulated at each time point compared to supernatant, however only 117 genes were invariably up-regulated at the 3 intracellular time points. These results indicate that genes over-expressed at a defined time point may be required at different stages of internalization or survival within macrophages or may represent a non-synchronization of the infection process even though all Typhi examined were internalized in the first 30 minutes. As an example, SPI-1 and SPI-2 TTSS have very different expression profiles, consistent with their respective function. The results obtained from microarray experiments were corroborated by qPCR. Two different technologies applied on different biological samples were consistent, supporting the validity of SCOTS-microarray analysis as an effective way to study host-pathogen interaction.

The molecular mechanism of typhoid fever has predominantly been indirectly derived from the Typhimurium murine typhoid fever model as these serovars share many virulence genes. Our data suggest that many virulence factors and global regulators common to both Typhi and Typhimurium are similarly up-regulated inside macrophages. The genes coding for the flagellar apparatus, chemotaxis, and iron transport systems were down-regulated by Typhi within macrophages, as described previously for Typhimurium (9), indicating that iron is available from the vacuole, which could explain why Typhi can afford to harbor many mutations in iron acquisition genes (3).

SPI-1 encodes a TTSS involved mainly in cell invasion and is an essential system for infection of epithelial cells of the gut (4). SPI-1 genes were down-regulated after bacterial internalization in macrophages as expected (Fig. 2A) and the expression profile of *prgI*, a SPI-1 encoded gene, was confirmed by qPCR (Fig. 3). The up-regulation of SPI-1 at the earliest times after invasion was not previously observed by Eriksson *et al* (9), since the earliest time point investigated in that study was four hours post-infection. It is interesting to note that SPI-1 and genes involved in motility have similar expression patterns (Fig. 2A, D), and transcription of flagella and SPI-1 genes are co-regulated by *fliZ* (32), which is consistent with our results. SPI-1 genes up-regulated during uptake of Typhi by macrophages (T0) may contribute to Typhi invasion of macrophages, as previously shown for Typhimurium (33). SPI-1 effector SopE, which is present in some Typhimurium strains, recruits Rab5 to the *Salmonella*-containing phagosome, promoting continuous fusion with early endosomes and prevents fusion with lysosomes (34). In addition, macrophage apoptosis is thought to be mediated

in part by SPI-1 effectors such as SipB (35). Thus, induction of SPI-1 during or immediately after invasion is not surprising. Another possible role of SPI-1 during macrophage infection was recently demonstrated by the loss of transposon mutants within SPI-1, such as *inv*, *sip* and *prg*, after passage into macrophages (36). We may also hypothesize that some other genes that are not currently known to be effectors may be translocated by the TTSS system of SPI-1 and may contribute to the initial interaction of Typhi with macrophages. Two genes encoding for unknown protein, STY1482 and STY1353, that were up-regulated by Typhi within macrophages, were recently identified as new TTSS effectors in Typhimurium (37) and named *steA* and *steC,* respectively. This confirms that our method can identify new virulence genes. Many cDNAs corresponding to genes with unknown functions or unique to Typhi were up-regulated inside human macrophages, including, STY1361-STY1367, STY2000-STY2002 and some SPI-6 genes, and will be important to consider for future studies to elucidate the intracellular lifestyle of this human specific pathogen.

Genes encoding the SPI-2 TTSS and the Mg^{2+} transport system (*mgtBC* located on SPI-3), were more highly expressed in vivo compared to supernatant, as was previously observed with Typhimurium inside murine macrophages (9). SPI-2 is well known to facilitate *Salmonella* survival inside macrophages (5, 6). Many genes encoded on SPI-2 were already up-regulated following bacterial uptake (T0), and other SPI-2 encoded genes were up-regulated only after 2 h of infection. Most of the SPI-2 encoding genes were over-expressed at all time points, consistent with their implication in creating and maintaining the *Salmonella* containing vacuole. Some SPI-2 genes were not differentially expressed, such as the *ttr* genes, which encode a tetrathionate reduction

system (38). In addition, transposon mutants of *ttr* genes of Typhimurium are not lost during infection of macrophages (36).

Most of the genes responsible for the SOS response were not differentially expressed by Typhi inside human macrophages compared to the supernatant. The SOS genes (*recA*, *umuCD*, *uvrABY*, *sulA* and *mutH*) were induced by Typhimurium inside murine macrophages (9). However, strong inductions of *dinI*, the repressor of the SOS response, as well as no induction of other DNA repair systems were observed. It is unclear if Typhi encounters oxidative stress inside human macrophages as systems involved to protect against oxidative stress, such as *oxyR* and *katG* were not differentially expressed (Fig. 2F). No clear induction of genes involved in acid response was observed in Typhi. It is possible that Typhi already experiences some acid or oxidative stress and that the SOS response was already activated by Typhi cells in contact with macrophages or their metabolic products, present in the supernatant, our in vitro control.

An important host defense mechanism involves production of antimicrobial peptides. Responses to antimicrobial peptides involved the *phoPQ* two-component system regulator, the *pmr* operon (39), *ugtL* (40), *pagP* (41), *pqaB* (42), *pgtE* (43), *virK* and its homologue *somA* (*ybjX*) (44) and *mig-14* (45, 46). These genes were up-regulated by Typhi in human macrophages (Fig. 2E). This implies that Typhi strongly responds to antimicrobial peptides inside the vacuole of human macrophages by modifying its lipid A, as previously described for Typhimurium (47). However, among all of these genes only *pgtE* up-regulation was identified by microarray analysis of gene expression of Typhimurium from murine macrophages (9). By contrast, most of the drug resistance

systems, like *marRAB, emrRAB, aac,* and *aadA,* were repressed or not differentially expressed by Typhi whereas these systems were up-regulated by Typhimurium (9).

Other differences in gene expression between Typhi and Typhimurium were also identified from the microarray data (Table S4). It is important to mention that the model from Erickson *et al.* (9) contains many technical differences from our model, including opsonization of bacterial cells, a higher multiplicity of infection, and number of host cells, a murine macrophage cell line (J774-A.1), a different in vitro control (cell culture medium), a different way to obtain cDNA (differential lysis) and the time course only overlapped at eight hours post-infection. Thus differences observed may be caused by one or more of these factors instead of the different serovar used.

There are two major regulators of *Salmonella* virulence genes required for survival in macrophages: *slyA* and *phoP* (8, 48-51). We detected significant induction of *phoP* and *slyA* in Typhi inside the macrophage, which was not seen in Typhimurium (9). A recent study identified 22 genes that were co-regulated by SlyA and PhoP in Typhimurium (52). Among these genes, 62% of them were significantly up-regulated by Typhi within macrophages, including well known virulence determinants such as the SPI-2 genes, *sseAB* and their regulator *ssrA,* and many genes involved in resistance to antimicrobial peptides. In contrast, Typhimurium expressed only 25% of these genes.

It was also proposed that once inside macrophages, Typhimurium uses gluconate and related carbohydrates as carbon sources (9). However, Typhi lacks the *dgo* operon involved in the utilization of this sugar and genes *gntT* and *gntU,* encoding transport

proteins, were not up-regulated in vivo. Repression of *gntU* was confirmed by using qPCR (Fig. 3). Other sugar transport systems (hexose, fructose) were also repressed. This suggests that Typhi uses a different carbon source inside macrophages than Typhimurium. Recent studies showed that isocitrate lyase, which allow the utilization of fatty acid as a carbon source via the glyoxylate bypass, is needed for persistence of *Mycobacterium tuberculosis* in human macrophages and Typhimurium in the mouse model (53, 54). The isocitrate lyase gene, *aceA* or *icl*, was previously found by SCOTS to be expressed at 48 h after infection of macrophages by two species of *Mycobacterium* (13, 14). In Typhi, the *aceA* gene was significantly induced starting at 8 h post-infection. In Typhimurium, *aceA* induction was not detected 12 h post-infection (9).

Our study demonstrates that by using SCOTS and microarray analysis, the transcriptome of intracellular bacteria can be obtained without altering the existing infection model. The method is an effective way to determine global bacterial gene expression profiling in the context of host infection. Its application on tissue biopsies from infected patients, as demonstrated with *Helicobacter pylori* in human biopsy samples (19), will be a potential means of further elucidating the bacterial genes expressed during infection of the human host. The SCOTS-cDNA mixture displayed an expected expression profile of Typhi virulence genes from infected macrophages. Our data suggest that inside THP-1 human macrophages, Typhi responds to antimicrobial peptides but does not encounter an acidic or oxidative environment, and that Typhi may use fatty acids as a carbon source as soon as 8 h after infection. Our global expression analysis identified many hypothetical as well as characterized Typhi genes that may contribute to adaptation and survival within macrophages, and such data are of

importance for future experimental studies to elucidate the intracellular lifestyle of this human-specific pathogen.

MATERIALS AND METHODS

Cell culture and bacterial infection model. The human monocyte cell line THP-1 (ATCC TIB-202) was maintained and infected as described previously (55). For RNA extraction, cells were seeded at 2 x 10^7 in 100 mm-wide tissue culture dishes. The wild-type virulent Typhi strain ISP1820 (obtained from D.M. Hone) was used as it is a well characterized strain that is sensitive to most antibiotics that are used for bacterial genetics, whereas CT18 is multidrug-resistant. The strain was grown at 37 ˚C overnight as a static culture in Luria-Bertani (LB) broth, to an O. D. $_{600}$ of 0.6 ($\approx$ 3 x 10^8 c.f.u./ml). Typhi cells were added to the THP-1 cell monolayer at a multiplicity of infection of 10 per cell, a low density that does not affect cell viability (data not shown). After incubation for 30 min at 37 ˚C, the supernatant was centrifuged and the pellet was lysed with TRIzol reagent (Invitrogen) and frozen at -70 ˚C. The infected cells were washed three times and incubated with complete RPMI initially containing 100 µg/ml of gentamicin, which was reduced to12 µg/ml after 2 h, to kill extracellular bacteria. At each time point, 0, 2, 8 and 24 h post-infection (p.-i.), macrophage monolayers were washed, lysed in TRIzol and store at -70 ˚C.

SCOTS. RNA from each condition was extracted according to the TRIzol reagent manufacturer's instructions (Invitrogen). RNA samples were then treated with RNase-

free DNase (Ambion). Each RNA sample was converted to cDNA as described previously (13, 55) (Fig. S5). Briefly, 5 μg of total RNA was converted to first strand cDNA by random priming, using a conserved primer containing a defined 5' end and random nonamer at the 3' end (16), with Superscript II (Invitrogen), according to the manufacturer's instructions. Second strand cDNA was synthesized using Klenow fragment (New England Biolabs) according to the manufacturer's instructions. Bacterial transcripts were separated from host cDNA by SCOTS, a selective hybridization to bacterial genomic DNA as described previously (13, 55). Captured cDNA was eluted, precipitated and amplified by PCR using the conserved primer added during reverse transcription. Noncompetitive PCR amplification of short random-primed DNA fragments with a single primer has previously been shown to yield a generally unbiased population of amplicons, even when applied to complex nucleic acid pools (56, 57). Three rounds of this capture hybridization were used to generate cDNA mixtures used as templates for microarray analysis.

***Salmonella* ORF array**. Typhi strain CT18 array description, labeling and hybridization conditions, data acquisition, and normalization are described elsewhere (27) (see A3). Bacterial genomic DNA was used as the reference channel on each slide to allow comparison of each time point and different samples (58). The log ratio (base 2) was used for statistical analysis using the ANOVA function with a standard Bonferonni correction by using TMev software (59). Data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession numbers GSE3094, GSE3095 and GSE3096.

Quantitative Real-Time PCR analysis. The results obtained from microarray experiments were corroborated by quantitative real-time PCR (qPCR) experiments on a new series of infection experiments (biological replicates). Infection and RNA extraction was performed as described above. cDNA was synthesized in triplicate using Superscript II (Invitrogen) with random hexamers (Sigma), according to the manufacturer's instructions. For each sample, a no reverse-transcriptase reaction served as a no template control. qPCR was performed using QuantiTect SybrGreen PCR Kit (Qiagen) according to the manufacturer's instructions. Primers are described in Table S5. For each qPCR run, the calculated threshold cycle (C_t) was normalized to the C_t of the internal control *rpoD* gene amplified from the corresponding sample and the fold-change was calculated as previously described (60). The alternative sigma factor *rpoD* was chosen as an internal control as no significant variation of expression for this gene in either Typhi (this study) or Typhimurium (9) was observed inside macrophages. In addition, expression of *rpoD* in *Escherichia coli* was shown to be independent of growth phase (61).

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REFERENCES

1. Parry, C. M., Hien, T. T., Dougan, G., White, N. J. & Farrar, J. J. (2002) *N. Engl. J. Med.* **347,** 1770-1782.
2. Marcus, S. L., Brumell, J. H., Pfeifer, C. G. & Finlay, B. B. (2000) *Microbes Infect.* **2,** 145-156.
3. Parkhill, J., Dougan, G., James, K. D., Thomson, N. R., Pickard, D., Wain, J., Churcher, C., Mungall, K. L., Bentley, S. D., Holden, M. T., *et al.* (2001) *Nature* **413,** 848-852.
4. Galan, J. E. (1999) *Curr. Opin. Microbiol.* **2,** 46-50.
5. Cirillo, D. M., Valdivia, R. H., Monack, D. M. & Falkow, S. (1998) *Mol. Microbiol.* **30,** 175-188.
6. Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vazquez-Torres, A., Gleeson, C., Fang, F. C. & Holden, D. W. (1998) *Mol. Microbiol.* **30,** 163-174.
7. Schwan, W. R., Huang, X. Z., Hu, L. & Kopecko, D. J. (2000) *Infect. Immun.* **68,** 1005-1013.
8. Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5189-5193.
9. Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. (2003) *Mol. Microbiol.* **47,** 103-118.
10. Schwan, W. R. & Kopecko, D. J. (1997) *Adv. Exp. Med. Biol.* **412,** 277-278.

11. McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., *et al.* (2001) *Nature* **413,** 852-856.

12. Hinton, J. C., Hautefort, I., Eriksson, S., Thompson, A. & Rhen, M. (2004) *Curr. Opin. Microbiol.* **7,** 277-282.

13. Graham, J. E. & Clark-Curtiss, J. E. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 11554-11559.

14. Hou, J. Y., Graham, J. E. & Clark-Curtiss, J. E. (2002) *Infect. Immun.* **70,** 3714-3726.

15. Morrow, B. J., Graham, J. E. & Curtiss, R., 3rd (1999) *Infect. Immun.* **67,** 5106-5116.

16. Daigle, F., Graham, J. E. & Curtiss, R., 3rd (2001) *Mol. Microbiol.* **41,** 1211-1222.

17. Faucher, S. P., Curtiss, R., 3rd & Daigle, F. (2005) *Infect. Immun.* **73,** 5217-5221.

18. Baltes, N. & Gerlach, G. F. (2004) *Infect. Immun.* **72,** 6711-6716.

19. Graham, J. E., Peek, R. M., Jr., Krishna, U. & Cover, T. L. (2002) *Gastroenterology* **123,** 1637-1648.

20. Dozois, C. M., Daigle, F. & Curtiss, R., 3rd (2003) *Proc. Natl. Acad. Sci. USA* **100,** 247-252.

21. Talaat, A. M., Lyons, R., Howard, S. T. & Johnston, S. A. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 4602-4607.

22. Boyd, E. F., Porwollik, S., Blackmer, F. & McClelland, M. (2003) *J. Clin. Microbiol.* **41,** 3823-3828.

23. Blanc-Potard, A. B. & Groisman, E. A. (1997) *Embo J.* **16,** 5376-5385.

24. Bueno, S. M., Santiviago, C. A., Murillo, A. A., Fuentes, J. A., Trombert, A. N., Rodas, P. I., Youderian, P. & Mora, G. C. (2004) *J. Bacteriol.* **186,** 3202-3213.

25. Zhang, X. L., Tsui, I. S., Yip, C. M., Fung, A. W., Wong, D. K., Dai, X., Yang, Y., Hackett, J. & Morris, C. (2000) *Infect. Immun.* **68,** 3067-3073.

26. Sharma, A. & Qadri, A. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 17492-17497.

27. Porwollik, S., Frye, J., Florea, L. D., Blackmer, F. & McClelland, M. (2003) *Nucleic Acids Res.* **31,** 1869-1876.

28. Park, Y. K., Bearson, B., Bang, S. H., Bang, I. S. & Foster, J. W. (1996) *Mol. Microbiol.* **20,** 605-611.

29. Lin, J., Smith, M. P., Chapin, K. C., Baik, H. S., Bennett, G. N. & Foster, J. W. (1996) *Appl. Environ. Microbiol.* **62,** 3094-3100.

30. Audia, J. P., Webb, C. C. & Foster, J. W. (2001) *Int. J. Med. Microbiol.* **291,** 97-106.

31. Rhen, M. & Dorman, C. J. (2005) *Int. J. Med. Microbiol.* **294,** 487-502.

32. Iyoda, S., Kamidoi, T., Hirose, K., Kutsukake, K. & Watanabe, H. (2001) *Microb. Pathog.* **30,** 81-90.

33. Cherayil, B. J., McCormick, B. A. & Bosley, J. (2000) *Infect. Immun.* **68,** 5567-5574.

34. Mukherjee, K., Parashuraman, S., Raje, M. & Mukhopadhyay, A. (2001) *J. Biol. Chem.* **276,** 23607-23615.

35. Hersh, D., Monack, D. M., Smith, M. R., Ghori, N., Falkow, S. & Zychlinsky, A. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2396-2401.

36. Chan, K., Kim, C. C. & Falkow, S. (2005) *Infect. Immun.* **73,** 5438-5449.

37. Geddes, K., Worley, M., Niemann, G. & Heffron, F. (2005) *Infect. Immun.* **73,** 6260-6271.

38. Hensel, M., Hinsley, A. P., Nikolaus, T., Sawers, G. & Berks, B. C. (1999) *Mol. Microbiol.* **32,** 275-287.

39. Gunn, J. S., Ryan, S. S., Van Velkinburgh, J. C., Ernst, R. K. & Miller, S. I. (2000) *Infect. Immun.* **68,** 6139-6146.

40. Shi, Y., Cromie, M. J., Hsu, F. F., Turk, J. & Groisman, E. A. (2004) *Mol. Microbiol.* **53,** 229-241.

41. Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M. & Miller, S. I. (1998) *Cell* **95,** 189-198.

42. Baker, S. J., Gunn, J. S. & Morona, R. (1999) *Microbiology* **145 (Pt 2),** 367-378.

43. Guina, T., Yi, E. C., Wang, H., Hackett, M. & Miller, S. I. (2000) *J. Bacteriol.* **182,** 4077-4086.

44. Detweiler, C. S., Monack, D. M., Brodsky, I. E., Mathew, H. & Falkow, S. (2003) *Mol. Microbiol.* **48,** 385-400.

45. Brodsky, I. E., Ernst, R. K., Miller, S. I. & Falkow, S. (2002) *J. Bacteriol.* **184,** 3203-3213.

46. Brodsky, I. E., Ghori, N., Falkow, S. & Monack, D. (2005) *Mol. Microbiol.* **55,** 954-972.

47. Gibbons, H. S., Kalb, S. R., Cotter, R. J. & Raetz, C. R. (2005) *Mol. Microbiol.* **55,** 425-440.

48. Libby, S. J., Goebel, W., Ludwig, A., Buchmeier, N., Bowe, F., Fang, F. C., Guiney, D. G., Songer, J. G. & Heffron, F. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 489-493.

49. Watson, P. R., Paulin, S. M., Bland, A. P., Libby, S. J., Jones, P. W. & Wallis, T. S. (1999) *Infect. Immun.* **67,** 4950-4954.

50. Miller, S. I., Kukral, A. M. & Mekalanos, J. J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5054-5058.

51. Bijlsma, J. J. & Groisman, E. A. (2005) *Mol. Microbiol.* **57,** 85-96.

52. Navarre, W. W., Halsey, T. A., Walthers, D., Frye, J., McClelland, M., Potter, J. L., Kenney, L. J., Gunn, J. S., Fang, F. C. & Libby, S. J. (2005) *Mol. Microbiol.* **56,** 492-508.

53. McKinney, J. D., Honer zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr. & Russell, D. G. (2000) *Nature* **406,** 735-738.

54. Fang, F. C., Libby, S. J., Castor, M. E. & Fung, A. M. (2005) *Infect. Immun.* **73,** 2547-2549.

55. Daigle, F., Hou, J. Y. & Clark-Curtiss, J. E. (2002) *Methods Enzymol.* **358,** 108-122.

56. Teng, D. H., Hsu, F., Peterson, I., Cardon, K. E., Caponigro, G. & Kamb, A. (2001) *Biotechniques* **30,** 868-877.

57. Ko, M. S., Ko, S. B., Takahashi, N., Nishiguchi, K. & Abe, K. (1990) *Nucleic Acids Res.* **18,** 4293-4294.

58. Talaat, A. M., Howard, S. T., Hale, W. t., Lyons, R., Garner, H. & Johnston, S. A. (2002) *Nucleic Acids Res.* **30,** e104.

59. Saeed, A. I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., *et al.* (2003) *Biotechniques* **34,** 374-378.

60. Livak, K. J. & Schmittgen, T. D. (2001) *Methods* **25,** 402-408.

61. Jishage, M. & Ishihama, A. (1995) *J. Bacteriol.* **177,** 6832-6835.

FIGURE LEGENDS

Figure 1. Scan of the first subarray of the microarray slides hybridized with cDNA, 1X SCOTS, 2X SCOTS and 3X SCOTS from 2h, from left to right. A) Cy5 (cDNA) signal only and B) both Cy5 (cDNA) and Cy3 (genomic DNA) signal. C) Number of genes detected following each round of SCOTS. A set of 100 Typhimurium-specific genes were used to calculate the detection threshold. Genes with signals greater than the median of these 100 genes plus 3 standard deviations were considered to be detected.

Figure 2. Number of genes (%) from selected functional groups that were significantly induced (■) or repressed (o). See text for details. AMPR, antimicrobial peptide resistance.

Figure 3. Real-Time qPCR (light color) and microarray (dark color) results for a set of 8 genes, compared to supernatant. See text for details.

FIGURES

Figure 1.

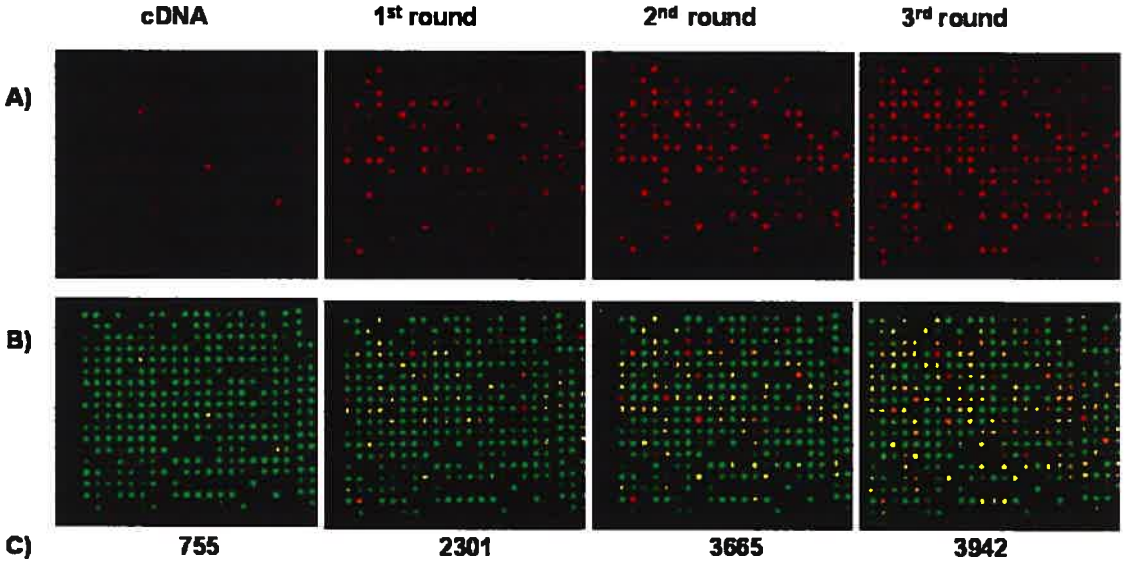


Figure 2.

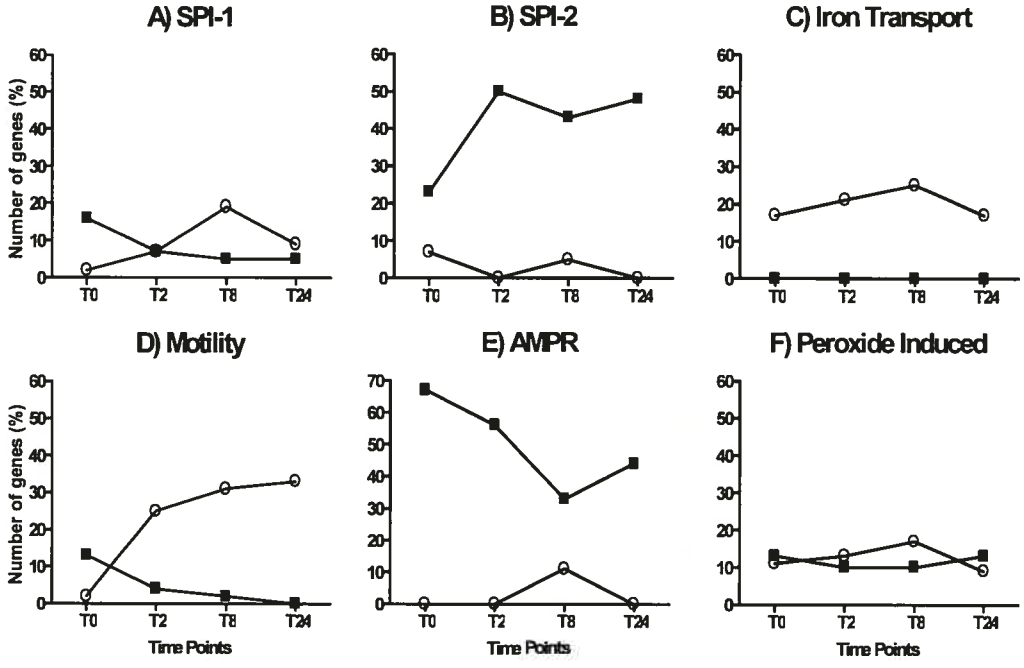
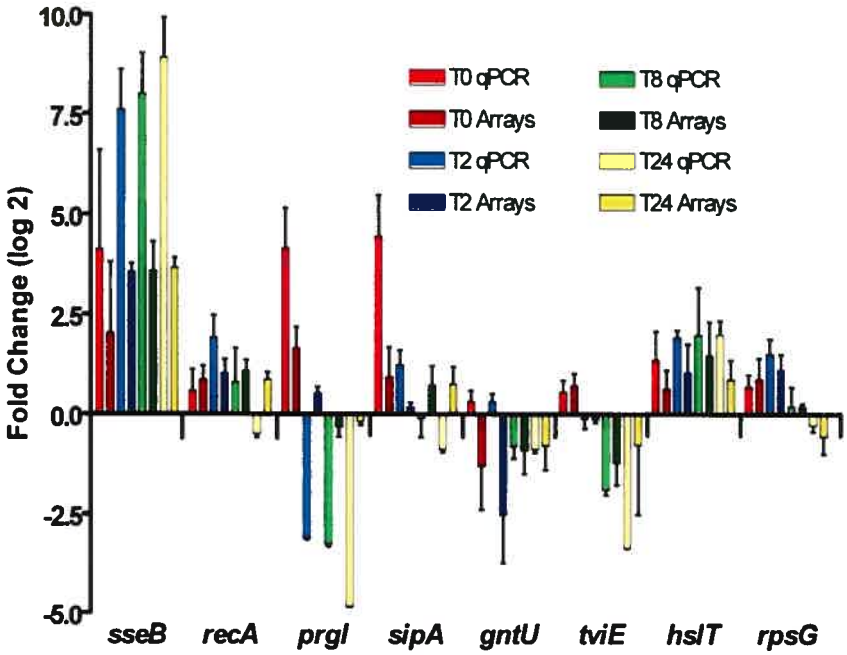


Figure 3.


10.0
7.5
5.0
2.5
0.0
-2.5
-5.0
Fold Change (log 2)
T0 qPCR
T0 Arrays
T2 qPCR
T2 Arrays
T8 qPCR
T8 Arrays
T24 qPCR
T24 Arrays
sseB
recA
prgI
sipA
gntU
tviE
hslT
rpsG

Préface au Chapitre 5

Le transcriptome de Typhi dans les macrophages humains a permis d'identifier une région absente du génome de Typhimurium (*STY1498-STY1499*) dont les deux gènes étaient fortement exprimés. Ces gènes uniques à Typhi pourraient être impliqués dans la virulence de Typhi et dans son interaction avec les macrophages humains. Le chapitre 5 décrit la caractérisation des gènes *clyA* et *taiA* codés dans cette région. Ces gènes sont impliqués dans l'interaction de Typhi avec les macrophages humains et sont des nouveaux facteurs de virulence spécifiques à Typhi. Cet article sera soumis prochainement au journal « Molecular Microbiology ».

J'ai effectué toutes les expériences décrites dans ce manuscrit et analysé tous les résultats. Ce manuscrit a été écrit par moi et révisé par France Daigle.

CHAPITRE 5, 4e article

The novel secreted invasin TaiA and the hemolysin ClyA are co-transcribed and have complementary functions that may help *Salmonella enterica* serovar Typhi persistence.

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Running title: Typhi-specific virulence genes

Keys Words: Typhi, ClyA, secreted protein, virulence, persistence, phagocytosis

ABSTRACT

Salmonella enterica contains many serovars that cause different diseases in humans. Serovar Typhimurium usually causes gastroenteritis while serovar Typhi causes a human restricted systemic infection called typhoid fever. These serovars share many virulence factors such as two different Type Three Secretion Systems (T3SS). However, differences in pathogenicity underlie that they also possess and use serovar-specific virulence factors. We have identified a Typhi genomic region encoding two open reading frames (ORF), STY1498 and STY1499, that are expressed during infection of human macrophages. STY1498 corresponds to *clyA*, which encodes a pore-forming hemolysin and STY1499 encodes a 27 KDa protein, without any attributed function, which we have named TaiA (Typhi associated invasin A). Transcriptional analysis revealed that these ORFs are part of an operon. In order to evaluate the implication of these genes in Typhi pathogenesis, isogenic Typhi strains harboring a non polar mutation of either *clyA* or *taiA* were constructed. Deletion of *clyA* enhanced Typhi survival at 48 h post-infection of macrophages but was not involved in cytotoxicity of macrophages. However, ClyA was cytotoxic for epithelial cells. Deletion of *taiA* reduced phagocytosis by human macrophages compared to the isogenic wild-type strain, but did not affect the survival rate in macrophages and was not required during infection of epithelial cells. Then, we have demonstrated that TaiA was a novel secreted protein. TaiA was secreted in medium inducing the invasion phenotype, but independently of the SPI-1 T3SS, which translocates proteins involved in the invasion of host cells. Our results revealed that products of this gene cluster unique to Typhi increase uptake by macrophages but reduced long-term bacterial survival. Those phenotypes might be

involved in promoting the use of macrophages as a stealth 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 infection, *S. enterica* is a major human pathogen. Out of the 2000 serovars of *S. enterica,* only a small fraction is associated with human infections (56). For example, serovars Typhimurium and Enteritidis cause a localized infection, gastroenteritis, in humans. Typhi causes a severe systemic infection called typhoid fever, which kills an estimated 600 000 people annually (53). 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 infection by *Salmonella*.

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 (20, 40). *S. enterica* possess a second T3SS located on SPI-2 which is required for survival inside macrophages and the infection of mammalian hosts (46, 48). The T3SS inject bacterial proteins directly inside the host cell and disturb their 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 initiate cell invasion (2, 36). Invasion of non-phagocytic host cells is strictly dependent on actin

rearrangement, and many SPI-1 T3SS secreted effectors affect actin polymerization, including SopE and SopE2 (24, 68). SPI-2 T3SS genes are induced by low concentrations of magnesium, phosphate and an acidic pH (4, 9, 12). SPI-2 translocated effectors are involved in modification of the *Salmonella* containing vacuole (SCV) and inhibition of lysosome fusion (32).

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 (8). 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 (65). Therefore, Typhi is likely to possess and use unique virulence factors to systemically infect humans. For example, Typhi is able to reduce intestinal inflammation by the production of Vi, an exopolysaccharide capsule which decreases the production of IL-8 and IL-17 by intestinal cells (57, 58, 59). Interestingly, Vi capsule is consistently absent in serovars that cause gastroenteritis in humans (56). Typhi is able to survive better inside human macrophages than Typhimurium (66). However, Typhimurium survives better inside murine macrophages than Typhi (66). The highest level of survival in macrophages seems to correlate with the host in which each serovars are able to cause a systemic infection. Survival in host macrophages is known to have a great impact on virulence (18) and host macrophages have been shown to be the reservoir of *Salmonella* during systemic disease (60). 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 mechanisms of pathogenicity.

Transcriptomic studies of Typhi identified a group of 117 genes constitutively induced within infected human macrophages (17). This group includes many genes of the SPI-2 T3SS, several genes involved in antimicrobial peptide resistance and many genes with no associated function during infection (17). Some of these genes were absent from the Typhimurium genome, including *clyA* (STY1498) and STY1499, which are two contiguous genes. ClyA also known as HlyE or SheA, is a well characterized hemolysin found in serovar Typhi, Paratyphi A and in some *Escherichia coli* strains (50). ClyA monomers are exported in outer membrane vesicles (OMVs). Differences in redox status in OMV compared to periplasm trigger ClyA oligomerization and insertion into the OMV membrane (71, 75). *E. coli* cells expressing *clyA* are cytotoxic for mammalian cells in a contact dependent manner (33, 49). Moreover, purified *E. coli* ClyA and OMVs containing ClyA have been shown to be cytotoxic for cultured epithelial cells and macrophages (33, 75). Typhi strains are not hemolytic on blood agar and *clyA* is not expressed in this growth condition (50). However, the vaccinal strain Ty21a has a hemolytic phenotype on blood agar plates and it has been suggested that expression of *clyA* by this strain may be caused by an aberrant regulation due to an unknown mutation (50). A specific antibody response toward ClyA during human infection by Typhi or Paratyphi has been reported recently, which indicates that ClyA is expressed *in vivo* (74). However, direct evidence of ClyA playing a role in Typhi pathogenesis has not been reported. STY1499 is a putative ORF of unknown function, that we have named *taiA* (Typhi Associated Invasin A). Expression of this gene cluster inside human macrophages suggests that these genes might be involved 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.

RESULTS

Presence of *clyA* and *taiA* in bacterial genome. *clyA* and *taiA* (*STY1499*) are inserted between *osmC* and STY1501 in Typhi (Fig. 1A) and are absent in Typhimurium, where *osmC* and STM1562 (homologue of STY1501) are contiguous. By comparative genomic hybridization, *taiA* and *clyA* were detected in typhoidal serovars Typhi and Paratyphi A (56). Interestingly, these genes are also absent in all tested strains of Enteritidis and Typhimurium, which cause gastroenteritis in humans (56). Serovars Montevideo, Oranienburg, Sendai, Javiana and Muenchen were also shown to possess these genes, although only one strain per serovar was tested (56). BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) analysis using the sequence encoding both genes reveals that this gene cluster was present in *Salmonella enterica* serovar Typhi, Paratyphi A, Javiana and Schwarzengrund but absent in other sequenced microbial genome. This gene cluster possesses a GC content of 40% which is relatively low compared to the average 52% of the genome of Typhi (52). The flanking genes, *osmC* and *STY1501*, possess a GC content of 52% and 45% respectively. *clyA* is present in many pathogenic *E. coli* strains and in the laboratory strain K-12 (38). BLAST analysis using the nucleotide sequence reveals that ClyA is also present in *Shigella flexneri* serotype 2a and 5. However, *taiA* was absent from *E. coli* and BLAST analysis using the nucleotide sequence did not identify any homologous genes. However, in the coliBASE database (http://colibase.bham.ac.uk/), the only orthologue of *taiA* was *Yersinia*

enterocolitica ORF 3776 (56% identity, 76% similarity). BLAST analysis at the protein level revealed homology to a hypothetical protein of *Y. enterocolitica* (YE2885) and to a putative signal transduction histidine kinase of *Y. bercovieri*. However, TaiA does not possess any known domain and it is unlikely that TaiA has any kinase activity. PSORTb (21) was used to find a putative subcellular localization of TaiA. Unfortunately, PSORTb was unable to predict a subcellular localization and no signal peptide for secretion was found.

***clyA* and *taiA* are arranged in an operon.** In order to identify possible promoters for *taiA* and *clyA* the NNPP version 2.2 was used with the prokaryote settings (www.fruitfly.org/seq_tools/promoter.html). A putative promoter sequence with a high score was detected 200 bp upstream of *taiA*. However, only low score promoters were found upstream of *clyA*. This suggests that *clyA* might be 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 was amplified with primers 1499-F and 1498-R which encompasses genes *taiA* and *clyA* (Fig. 1B). Therefore, *taiA* and *clyA* are co-transcribed and belong to the same operon.

***clyA* and *taiA* are expressed in human macrophages.** Transcriptomic study of Typhi infecting human cultured macrophages (THP-1) revealed that *taiA* and *clyA* were induced during infection (17). 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 contains RNA from bacteria that were not associated with macrophages and was used as the

control condition, as previously described for the microarray experiment (17). The 0 h time point contains RNA from bacteria associated with macrophages, either extracellularly or intracellularly. Both genes seem to be strongly induced when the bacteria are associated with macrophages (0 h) and even more at later time points during the infection. Interestingly, both genes have the same expression pattern, which corroborate the result indicating that these genes are co-transcribed.

ClyA reduced Typhi survival in human macrophages. Previous study of Typhi *clyA* did not identify a condition that induced its expression (50). However, our data clearly shows that *clyA* was expressed during interaction with human macrophages, suggesting that ClyA may have a role during infection of host cells. Therefore, a strain containing a non-polar deletion of *clyA* was constructed. The contribution of *clyA* in uptake by or survival in human cultured macrophages (THP-1) was investigated using a gentamicin protection assay. The *clyA* deletion did not affect bacterial uptake significantly but enhanced survival of Typhi in human macrophages by 40% ($P = 0.02$) of the wild-type phenotype, after 48 h of infection (Fig. 2A). To validate this result, the *clyA* gene was cloned on a low copy plasmid and transformed in the Δ*clyA* to complement the mutation and in the wild-type strain as a control. These strains had the same survival rate in macrophages, which demonstrates that the observed phenotype of the Δ*clyA* strain was due to the mutation it harbored (Fig. 2A). In order to investigate the effect of ClyA production inside macrophages, a cytotoxic assay was performed by monitoring the release of LDH, a cytolysis indicator. Macrophages infected with the Δ*clyA* strain released the same amount of LDH as the macrophages infected by the wild-type strain (Fig. 2B).

ClyA promotes lysis of human epithelial cells. A previously published study reported that purified *E. coli* ClyA is cytotoxic for epithelial cells (75). It is currently unknown if Typhi ClyA is cytotoxic for epithelial cells, in the context of bacterial-host interaction. Therefore, we tested the effect of a non-polar deletion of *clyA* on infection of human epithelial cells. Adhesion, invasion, and cytotoxic assays were performed. The Δ*clyA* strain showed the same adhesion level as the wild-type strain (data not shown). However the mutant strain was 60% more invasive ($P = 0.03$) than the wild-type strain (Fig. 2A). The mutant strain was 25% less cytotoxic ($P = 0.001$) to epithelial cells than the wild-type strain (Fig. 2B). Complementation restored the wild-type phenotype.

TaiA is involved in bacterial uptake by human cells. Even if no similarity with any known virulence protein was identified for *taiA*, its expression profile during infection of human macrophages suggests that TaiA might be involved in Typhi interaction with human cells. To test this hypothesis, a mutant strain harboring a non-polar deletion of *taiA* was constructed. Deletion of *taiA* reduced bacterial uptake by or association with human macrophages to 60% ($P = 0.00006$) of the wild-type value but had no effect on bacterial survival inside human macrophages (Fig. 3A). Complementation of the *taiA* mutant with a wild-type copy of the gene on a low-copy plasmid restored the wild-type phenotype. In order to differentiate between bacterial association and invasion, macrophages were pretreated with cytochalasin D before infection. Cytochalasin D is an inhibitor of host cell cytoskeletal function which blocks bacterial uptake by macrophages (61). 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 the level of the wild-type (Fig. 3B). Taken together, these results suggest that TaiA might be involved in increasing bacterial uptake by host macrophages but is not involved in bacterial association with macrophages. Adherence to and invasion of epithelial cells were then investigated. The Δ*taiA* strain had the same level of adherence to (data not shown) and invasion of epithelial cells as the wild-type strain (Fig. 3C). Therefore, deletion of *taiA* did not seem to impair Typhi interaction with epithelial cells. To investigate the effect of additional copy of *taiA*, the plasmid for complementation (pWSK*taiA*) was introduced in the wild-type strain and the adherence to and invasion level of HeLa cells were compared to the levels of the wild-type strain harboring the empty vector (pWSK29). No difference was seen for the adherence level. However, the wild-type strain harboring pWSK*taiA* was 5 times more invasive (P = 0.01) than the wild-type strain harboring the vector alone (Fig. 3C). In addition, the Δ*taiA* strain harboring pWSK*taiA* was 2.5 times more invasive (P = 0.01) than the mutant strain harboring the vector alone (Fig. 3C). This indicates that additional copy of *taiA* enhanced invasion of HeLa cells by Typhi. Moreover, this effect seems to be directly proportional to the copy number of *taiA*, since the wild-type strain harboring pWSK*taiA* is more invasive than the mutant strain harboring pWSK*taiA*. Thus, TaiA seems to be involved in the uptake by host cells. 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.

TaiA is a novel secreted protein. Because TaiA seems to affect host cells during infection, we hypothesized that TaiA might be secreted. To allow detection of TaiA by western blotting, the protein was tagged at its C-terminal with 2 HA epitopes and cloned

in a low copy vector with its native promoter. The HA tag has been proven useful to study protein secretion and C-terminal tags are usually well tolerated (72). Moreover, this tag was previously used to monitor the secretion of many SPI-2 effectors, such as PipB, SseJ and SseL by Typhimurium T3SS (10, 31, 34). The presence of TaiA-2HA in the supernatant of bacteria grown in condition known to induce expression of genes involved in invasion of host cells (36). A 29 KDa proteins was detected by anti-HA antibody in the supernatant fraction and in the pellet fraction of strain harboring p*taiA*-2HA but not of the strain harboring the vector alone (Fig. 4, line 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 neither a SPI-1 nor a SPI-2 T3SS secreted effector. Many virulence factors of *Salmonella* that interfere with host cell function are secreted by T3SS and invasion of host cells is associated with SPI-1 T3SS. Moreover, there are many reports showing that *Salmonella* SPI-1 T3SS is also used to secrete proteins in contact with macrophages. For example, SPI-1 T3SS is known to induce macrophage cell death by injecting SipB (7, 27). As for SPI-2 T3SS, SPI-1 T3SS is also able to translocate proteins encoded outside SPI-1, such as SopE and SopE2 (24, 68). Because TaiA seems to have a function in early interaction with human macrophages and because it is secreted in SPI-1 inducing condition (LB-NaCl), it seems possible that TaiA is secreted by SPI-1 T3SS. A possible secretion by SPI-1 T3SS was investigated by monitoring secretion of TaiA-2HA in a Δ*invA* strain, which is unable to assemble a functional SPI-1 T3SS (69). TaiA-2HA was

still secreted by a Δ*invA* strain, suggesting that its secretion in SPI-1 inducing media was not dependent of SPI-1 T3SS (Fig. 4, line 3).

TaiA was not secreted by SPI-1 T3SS and because it was involved in Typhi interaction with human macrophages, it seems possible that it may be secreted by SPI-2 T3SS, which is used to translocate bacterial effectors into host macrophages. Moreover, Pavlidis Template Matching analysis using the Tigr MeV software (64) of Typhi transcriptomic data inside THP-1 cells revealed that *taiA* exhibited the same expression profiles as some SPI-2 effectors, such as *sseG* (17 , 23). Some SPI-2 T3SS effectors are encoded outside SPI-2, such as SseL and SifA (5, 10, 63). Therefore the secretion of TaiA by SPI-2 T3SS was investigated. LPM pH 5.8 medium has been shown to stimulate secretion of SPI-2 secreted effectors (9). Under this condition, a 29 KDa protein, detected by anti-HA tag antibody, was only produced by bacteria harboring p*taiA*-2HA but not the vector alone (Fig. 5A). However, this protein was detected only in the bacterial pellet but not in the culture supernatant (Fig. 5A). As a control for SPI-2 secretion, SseB was detected in the culture supernatant of Typhi strain harboring the vector alone or p*taiA*-2HA. GroEL was used as a control of bacterial cytoplasm leakage and was not detected in the culture supernatant. Secretion studies performed using N salts medium containing 8 μM $MgCl_2$, a condition which was also shown to induce expression of SPI-2 T3SS (12), failed to detect secretion of TaiA-2HA (data not shown). Our data clearly show that TaiA is not secreted under SPI-2 inducing condition.

TaiA-2HA was detected in the bacterial pellet and since *taiA*-2HA was cloned with its native promoter, this means that TaiA was produced under SPI-2 inducing

conditions but not secreted. Induction of SPI-2 T3SS structural and effector genes required the *ssrA/ssrB* two component system (26, 77). To investigate a possible regulation of *taiA* by SsrB, we compared expression of *taiA* in the wild-type and in a Δ*ssrB* isogenic strain, 24 h post-infection of human macrophages. qPCR results reveal that expression of *taiA* and *clyA* in Δ*ssrB* strain was almost the same as in the wild-type strain (Fig. 5B). However, SPI-2 encoded effector *sseB* was strongly repressed in a Δ*ssrB* strain, as expected. Thus, *taiA* and *clyA* are not part of the SsrB regulon and TaiA is not secreted by SPI-2 T3SS.

TaiA enhance invasion of HeLa cells by *E. coli*. As TaiA was involved in Typhi uptake by host cells, we next investigate if *taiA* may confer an invasion phenotype to *E. coli*, which is unable to invade epithelial cells. Therefore, pWSK*taiA* and pWSK29 were transformed in *E. coli* DH5α and invasion assay were performed with those strains. *E. coli* harboring pWSK*taiA* was 2 times more invasive ($P = 0.03$) than *E. coli* harboring the empty vector (Fig. 6). This confirm that TaiA is not secreted by neither T3SS, since they are absent in *E. coli* DH5α, and is involved in bacterial uptake by host cells.

DISCUSSION

The goal of this study was to assess the implication of a gene cluster up-regulated during macrophage infection and specific to typhoidal *Salmonella* in Typhi interaction with host cells. One may expect that serovar-specific virulence factors could be involved in host and disease specificity. *Salmonella* serovars are thought to have evolved by acquisition of genomic region encoding virulence factors by horizontal gene transfer

(HGT) (22 , 25, 40) or by genome degradation, such as deletion of genes or pseudogene formation (41). Gene clusters acquired by HGT are usually AT-rich, compared to the average GC content of *Salmonella* genomes, and H-NS tightly regulates their expression (37). For example, there are evidences that SPI-1 and SPI-2 T3SS have been acquired by HGT, which correlates with extensive binding of H-NS in those DNA regions (37 , 47). A recent study identified *taiA* (*STY1499*) as a putative horizontally acquired gene, present only in typhoidal *Salmonella*, not in gastroenteritis mediating *Salmonella* (73). Consistent with this, a comparative genomic hybridization (CGH) study revealed that *taiA* and *clyA* are present together in all Typhi, Paratyphi A, Montevideo, Oranienburg, Sendai, Javiana strains tested and in one serovar Muenchen strain. Interestingly, these serovars are clustered together in the *S. enterica* genovar tree based on the CGH data, which suggest that they are genetically closely related (56). By contrast the gastroenteritis-associated serovars Typhimurium and Enteritidis, which do not contain *taiA* nor *clyA*, are clustered together in a distant branch of the CGH based genovar tree.

Transcriptional analysis revealed 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 (17). In this study both *taiA* and *clyA* were clustered with many SPI-2 encoded or SPI-2 translocated effector coding gene. This lead us to question if *taiA* and *clyA* were regulated by SsrB. The SsrA/SsrB two component system is directly involved in the induction of SPI-2 T3SS structural genes and secreted effectors (10, 26, 77). SsrB also regulates a number of

other genes outside SPI-2 (78). However, our results suggest that SsrB does not regulate expression of *taiA* nor *clyA* (Fig. 5B).

Typhi ClyA contributed to be cytotoxicity in epithelial cells (Fig. 2B), as was shown for *E. coli* ClyA (75). 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 increase survival rate, without affecting cytotoxicity (Fig. 2). The mechanisms underlying differences in the interaction of ClyA with epithelial cells and macrophages are currently unknown. The gentamicin used in the gentamicin protection assay kills extracellular bacteria that have not invaded host cells or that have been released from host cells following host cell death. In theory, a lower level of cytotoxicity toward host cells would likely result in a lower release of bacteria, thus enhancing the apparent survival of bacteria in this model. However, in the case of the Δ*clyA* strain in macrophages, enhanced survival cannot be explained by a decrease in macrophage lysis, because macrophages infected with the wild-type or the mutant strain showed no differences in cell viability.

Inside macrophage, it is possible that ClyA forms pore within the SCV, which may alter the SCV content and affect survival of Typhi. Insertion of *E. coli* ClyA in epithelial cell outer membrane results in intracellular oscillation of Ca^{2+} concentrations (67). SPI-1 T3SS insertion in epithelial cell membrane has been shown to increase the rate of fusion of the SCV with lysosomes via a Ca^{2+}-dependent pathway, which compromises bacterial growth (62). It is currently unknown if SCV perforation by SPI-2

T3SS induces host functions that reduces bacterial growth (70). The ClyA pore is at least 35 Å wide, which is sufficiently large to allow passage of small compounds like maltose (50, 71). One may therefore hypothesize that the formation of pores in SCV by ClyA could affect 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 the opposite.

Because, host macrophages are the reservoir of *Salmonella* during systemic infection (60), expression of *clyA* by Typhi inside human macrophages may lead to reduced bacterial growth and lead to persistent infection of human macrophages during the systemic phase of the infection. Growth control inside host cells by intracellular pathogens is a new concept, and some mechanisms have been reported (70). SciS, a Typhimurium homologue of *Legionella pneumophila* IcmF, has been shown to reduce intracellular growth in macrophages. Interestingly, loss of *sciS* attenuated virulence of Typhimurium in mice (54). Typhimurium mutants unable to trigger induction of host nitric-oxide synthase were found to overgrow in macrophages (16). Induction of host defense systems is not the only strategy used by *Salmonella* to control its intracellular growth. Coombes *et al* (11) reported that YdgT reduces SPI-2 T3SS expression during infection. A Δ*ydgT* strain displayed an increased survival in macrophages but killed mice less efficiently than the wild-type strain. Controlled growth in host cells has been also linked to chronic infection (43, 70). A recent report compared the net growth rate in intestinal mucosa of pigs infected with serovar Cholerasuis and Typhimurium. In this model, Cholerasuis causes a systemic infection with a high mortality rate, while Typhimurium causes acute enteritis that is rarely fatal (55). They found that Cholerasuis replicated slower than Typhimurium in intestinal mucosa, which is consistent with the

necessity to control growth to cause a systemic infection. In the 129sv mouse line, Typhimurium causes a chronic asympomatic infection (42). In this model, host macrophages seem to be the reservoir of persistent *Salmonella* and the mean number of *Salmonella* per cells was about 3, which is an indication that *Salmonella* growth was controlled (42). This goes well with Typhi longer incubation period than Typhimurium.

Moreover, because the 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. 3A). Reduction in bacterial uptake does not seem to be caused by a reduction in bacterial adhesion or association to 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. 3B). 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. 3C). However, overexpressing *taiA* in Typhi or *E. coli* enhance its invasiveness (Fig. 3C and Fig. 6). In the wild-type Typhi strain, the SPI-1 T3SS is probably sufficient to trigger invasion of HeLa cells which cover-up the effect of TaiA. Nevertheless, our results indicate that TaiA is a novel invasin of Typhi.

Production of TaiA was observed in SPI-1 and SPI-2 inducing media, but secretion of TaiA was only detected in SPI-1 inducing conditions, not in SPI-2 inducing media (Fig. 4 and Fig. 5A). TaiA secretion was found to be independent of both T3SS by western blotting. Moreover, a confirmation that TaiA is not secreted by T3SS was

obtained by showing that TaiA enhanced invasion of HeLa cells by *E. coli* which does not possess SPI-1 or SPI-2 T3SS. It was surprising to note secretion of TaiA in SPI-1 inducing condition because it is usually not associated with function involved in interaction with host macrophages. However there are a number of studies implicating SPI-1 T3SS during infection of macrophages and SPI-1 translocated effectors are involved in *S. enterica* interaction with macrophages. For example, SPI-1 T3SS causes early macrophages apoptosis, a function attributed to SipB (7, 19, 27, 39). These studies suggest that SPI-1 T3SS inducing conditions exist during early interaction with human macrophages. However, TaiA might be secreted in other conditions and TaiA secretion might be regulated by an unknown mechanism. Detection of TaiA in the supernatant fraction or in the cell fraction at the initial time point of macrophage infection (T0) by western blotting was unsuccessful, probably because TaiA concentrations were 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 (71, 75), one may hypothesized that TaiA also use this export mechanism and that these proteins may interact together in the OMVs. These possibilities are currently under investigation.

Some *Salmonella* virulence factors have been previously identified to influence macrophage phagocytosis. For example, O-antigen side chains influence uptake by human macrophages (44), SPI-1 T3SS increases invasion of porcine macrophages by Typhimurium (6) and the type IVB pili enhanced uptake of Typhi by human macrophages (51). Moreover, Drecktrah *et al* (14) have demonstrated that the mechanism of *Salmonella* entry inside host macrophages affects the growth rate and

gene expression. Therefore, *Salmonella* virulence factors, like TaiA, SPI-1 T3SS, LPS and type IVB pili, may affect the mechanism of phagocytosis to promote survival and appropriate growth inside host macrophages.

This study shows that *taiA* and *clyA* are co-transcribed and seem to have complementary function. TaiA is a novel secreted invasin which increased bacterial uptake by human macrophages and ClyA reduced bacterial growth, which might result in an increased use of macrophages as a stealth environment. This in turn may promote persistent infection of the host which is a key feature of typhoid fever. Acquisition of this gene cluster by typhoidal *Salmonella* may explain in part their capacity to cause systemic infection in humans.

EXPERIMENTAL PROCEDURES

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 invasion assay or SPI-1 induction, bacteria were grown in LB containing 0.3 M NaCl (LB-NaCl) without aeration. For SPI-2 induction, low phosphate, low magnesium-containing medium (LPM) pH 5.8 was used as described (9). When required, antibiotics, amino acids or supplements were added at the following concentrations: ampicilin (Ap), diaminopimelic acid (DAP), 50 μg ml^{-1}; chloramphenicol (Cm), 34 μg ml^{-1}; tryptophan, cysteine and arginine, 22 μg ml^{-1}. Transformation of bacterial strains was routinely done

by using the calcium/manganese based (CCMB) or electroporation methods as described (45).

Generation of mutants and complementation. To generate non-polar mutations of *taiA* and *clyA,* the overlap-extension PCR method described by Basso *et al.* was used (3). For *taiA*, fragments were amplified with primers STY1499-BF and STY1499-BR for the 5' end of the gene and primers STY1499-EF and STY1499-ER for the 3' end of the gene. These 2 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 BamHI and XbaI and ligated into suicide vector pMEG375 (29). 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 (30). 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. To complement these mutants, *taiA* and *clyA* were cloned separately into the low copy vector pWSK29. This plasmid was shown to have no deleterious effect on Typhi infection of host cells (1). *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 BamHI and XbaI and ligated into pWSK29 resulting in plasmids pWSK*taiA* and pWSK*clyA*. pWSK*taiA* was transformed

in the wild-type strain and in DEF061 to complement the *taiA* mutation, resulting in strains DEF074 and DEF075 respectively. pWSK*clyA* was transformed in the wild-type strain and in DEF062 to produce DEF123 and DEF124 respectively.

Generation of mutant strains of SPI-1 and SPI-2 T3SS. Mutation 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 in pMEG-375, digested by BamHI and XbaI resulting in plasmid 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 an *ssrB* mutant.

Epitope tagging of TaiA. Primer 1499-R-2HA was designed to contain the last 22 nucleotides (without the stop codon) of *taiA* and 2 HA tag sequences. This primer was used with STY1499-FC to PCR amplify STY1499 with its native promoter and add 2 HA sequences at its 3' end. The resulting fragment was digested with XbaI and BamHI and ligated into pWSK29 to create 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 blot. p*taiA*-2HA was also transformed in the SPI-1 (DEF147) and SPI-2 (DEF149) mutants resulting in strains DEF169 and DEF171 respectively.

Infection of human cultured macrophages. The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 (Wisent) containing 10% fetal calf serum (FCS) (Invitrogen), 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7) (Wisent), 2 mM L-glutamine, 1 mM sodium pyruvate and 1% MEM (modified Eagle's medium) 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% 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 72 h. Bacteria were grown overnight standing in LB, which corresponds to an OD_{600} of 0.6 ($\approx 3 \times 10^8$ CFU) and were then added to the cell monolayer at a multiplicity of infection (MOI) of 10:1 and centrifuged for 5 min at 800 × *g* to synchronize phagocytosis. After incubation for 20 min at 37 °C, the infected cells were washed three times with prewarmed phosphate buffered saline (PBS), pH 7.4, and the infected monolayers were either lysed (T0) from the tissue culture dishes or incubated 2 h with supplemented media as above containing 100 μg ml^{-1} of gentamicin (Wisent) to kill extracellular bacteria. Then, the infected monolayers were washed three times with prewarmed PBS and further incubated for an additional 46 h (or 6 h and 22 h for RNA extraction) in the presence of fresh supplemented tissue culture medium containing 12 μg ml^{-1} of gentamicin. The infected cells were then washed three times with prewarmed PBS and the infected monolayers were lysed (T48). The cells were lysed by addition of 1 ml of 0.1% sodium deoxycholate in PBS (PBS-DOC) per well and the number of surviving bacteria were determined as colony forming units (CFU) by plating on LB agar. Percent phagocytosis was calculated by (CFU at T0/CFU in the initial inoculum of bacteria added to each well) × 100. The survival rate at 48 hours

corresponds to (CFU at T48/CFU at T0) × 100. Results are expressed as the mean ± standard error of the replicate experiments compared to wild-type value for each replicate. For complement study, the wild-type strain harboring the plasmid for complementation was used as the control. Student's T-test was used for statistical analysis. When 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 (61). The addition of cytochalasin D was maintained throughout the infection.

Infection of human cultured epithelial cells. HeLa cells (ATCC CCL-2) were grown in DMEM medium (Wysent) supplemented with 10% heat-inactivated FCS (Wysent) and 25 mM HEPES (Wysent) (complete medium). Infection was carried out as previously described (15). One day before infection, cells were seeded at 2.5×10^5 in 24-well tissue culture plates. An hour before infection, cells were washed 3 times with prewarmed PBS, and fresh complete medium was added to each well. Bacteria were grown overnight in LB-NaCl to an OD_{600} of 0.6 ($\approx 3 \times 10^8$ CFU) and added to each well at a MOI of 20 (100 for *E. coli*). The 24-well plates were then centrifuged at 800 × *g* for 5 min to synchronize infection, incubated at 37 °C in 5% CO_2 for 90 min and rinsed three times with PBS. Cells were either lysed with 1 mL of PBS-DOC to evaluate the level of adherence (T90) or incubated for a further 90 minutes with complete medium containing 100 μg ml^{-1} of gentamicin to kill extracellular bacteria and assess the invasion level (T180). Cells were then lysed as described above. Samples were diluted and spread on LB plates for enumeration by viable colony counts. The adherence level was calculated by (CFU at T90/CFU in the initial inoculum of bacteria added to each well) × 100. The invasion level corresponds to (CFU at T180/CFU at T90) × 100.

Results are expressed as the mean ± standard error of the replicate experiments compared to the wild-type value for each replicate. For complementation studies, the wild-type strain harboring the plasmid for complementation was used as the control. To evaluate the effect of overproduction of *taiA* during infection of HeLa cells, the wild-type strain harboring the empty vector was used as the control. Statistical differences were assessed using Student's T-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 3 h for HeLa cells, supernatants were collected and lactate dehydrogenase (LDH) release was evaluated with the Cytotox96 Kit (Promega) according to manufacturer's instruction. Percentage of cytotoxicity is expressed as 100 × ((experimental release / spontaneous release)$_{\text{test strain}}$ / (experimental release / spontaneous release)$_{\text{control strain}}$) in which spontaneous release is the amount of LDH activity in the supernatant of uninfected cells.

RNA Isolation, Reverse Transcriptase and Real-Time qPCR. RNA was isolated from bacteria in the supernatant of infection and at 0, 8 and 24 h post-infection of human macrophages (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 % EtOH and resuspended in diethyl pyrocarbonate-treated water. Rigourous 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 qPCR (qPCR) with primers 16s-F and 16s-R (data not shown). 50 ng of RNA was

reverse-transcribed by using Superscript II (Invitrogen) with 0.5 µg of random hexamers (Sigma). As a negative control, a reaction without Superscript II was also included (NRT). qPCR reactions were performed in a Rotor-Gene 3000 (Corbett Research) by using QuantiTect SYBR Green PCR kit (Qiagen), according to manufacturer's instructions. Primers 1499-F and 1499-R and primer 1498-F and 1498-R were used to amplify *taiA* and *clyA* respectively. Primers sseB-F and sseB-R were used to amplify *sseB* as a positive control to investigate SsrB regulation of *taiA* and *clyA*. The transcriptional level of the genes of interest in each condition was normalised against a reference gene (16S rRNA, primer 16s-F and 16sR) and analyzed by applying the $2^{-\Delta\Delta Ct}$ method (35). 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 LB-NaCl standing or in LPM pH 5.8 shaking to an OD_{600} of 0.6. Bacteria were pelleted by centrifugation at 12000 × *g* for 5 min at 4 °C. Supernatant was collected, filtered through a 0.2 µm syringe filter (Fisher) and then precipitated with trichloroacetic acid (10% final concentration, v/v) at 4 °C for 16 h. The trichloroacetic acid insoluble fraction was collected by centrifugation, washed 2 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% glycerol, 2% SDS, 0.05% β-mercaptoethanol, 0.05% bromophenol blue) according to the OD_{600} of the original culture. When necessary, samples were neutralized with 1 µL of 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% SDS-polyacrylamide gels and then transferred on PVDF

membranes by using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) according to 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 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) (9), 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).

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REFERENCES

1. **Abromaitis, S., S. Faucher, M. Beland, R. Curtiss, 3rd, and F. Daigle.** 2005. The presence of the *tet* gene from cloning vectors impairs *Salmonella* survival in macrophages. FEMS microbiology letters **242:**305-312.

2. **Altier, C.** 2005. Genetic and environmental control of *Salmonella* invasion. Journal of microbiology (Seoul, Korea) **43 Spec No:**85-92.

3. **Basso, H., F. Rharbaoui, L. H. Staendner, E. Medina, F. Garcia-Del Portillo, and C. A. Guzman.** 2002. Characterization of a novel intracellularly activated gene from *Salmonella enterica* serovar Typhi. Infection and immunity **70:**5404-5411.

4. **Beuzon, C. R., G. Banks, J. Deiwick, M. Hensel, and D. W. Holden.** 1999. pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella typhimurium*. Molecular microbiology **33:**806-816.

5. **Beuzon, C. R., S. Meresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot, and D. W. Holden.** 2000. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. The EMBO journal **19:**3235-3249.

6. **Boyen, F., F. Pasmans, E. Donne, F. Van Immerseel, C. Adriaensen, J. P. Hernalsteens, R. Ducatelle, and F. Haesebrouck.** 2006. Role of SPI-1 in the interactions of *Salmonella* Typhimurium with porcine macrophages. Veterinary microbiology **113:**35-44.

7. **Chen, L. M., K. Kaniga, and J. E. Galan.** 1996. *Salmonella spp.* are cytotoxic for cultured macrophages. Molecular microbiology **21:**1101-1115.

8. **Connor, B. A., and E. Schwartz.** 2005. Typhoid and paratyphoid fever in travellers. The Lancet infectious diseases **5:**623-628.

9. **Coombes, B. K., N. F. Brown, Y. Valdez, J. H. Brumell, and B. B. Finlay.** 2004. Expression and secretion of *Salmonella* pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL. The Journal of biological chemistry **279:**49804-49815.

10. **Coombes, B. K., M. J. Lowden, J. L. Bishop, M. E. Wickham, N. F. Brown, N. Duong, S. Osborne, O. Gal-Mor, and B. B. Finlay.** 2007. SseL is a *Salmonella*-specific translocated effector integrated into the SsrB-controlled *Salmonella* pathogenicity island 2 type III secretion system. Infection and immunity **75:**574-580.

11. **Coombes, B. K., M. E. Wickham, M. J. Lowden, N. F. Brown, and B. B. Finlay.** 2005. Negative regulation of *Salmonella* pathogenicity island 2 is required for contextual control of virulence during typhoid. Proceedings of the National Academy of Sciences of the United States of America **102:**17460-17465.

12. **Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel.** 1999. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. Molecular microbiology **31:**1759-1773.

13. **Dozois, C. M., F. Daigle, and R. Curtiss, 3rd.** 2003. Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic *Escherichia coli* strain. Proceedings of the National Academy of Sciences of the United States of America **100:**247-252.

14. **Drecktrah, D., L. A. Knodler, R. Ireland, and O. Steele-Mortimer.** 2006. The mechanism of *Salmonella* entry determines the vacuolar environment and intracellular gene expression. Traffic (Copenhagen, Denmark) **7:**39-51.

15. **Elsinghorst, E. A.** 1994. Measurement of invasion by gentamicin resistance. Methods in enzymology **236:**405-420.

16. **Eriksson, S., J. Bjorkman, S. Borg, A. Syk, S. Pettersson, D. I. Andersson, and M. Rhen.** 2000. *Salmonella typhimurium* mutants that downregulate phagocyte nitric oxide production. Cellular microbiology **2:**239-250.

17. **Faucher, S. P., S. Porwollik, C. M. Dozois, M. McClelland, and F. Daigle.** 2006. Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. Proceedings of the National Academy of Sciences of the United States of America **103:**1906-1911.

18. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. (USA) **83:**5189-5193.

19. **Fink, S. L., and B. T. Cookson.** 2006. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. Cellular microbiology **8:**1812-1825.

20. **Galan, J. E.** 1999. Interaction of *Salmonella* with host cells through the centisome 63 type III secretion system. Current opinion in microbiology **2:**46-50.

21. **Gardy, J. L., M. R. Laird, F. Chen, S. Rey, C. J. Walsh, M. Ester, and F. S. Brinkman.** 2005. PSORTb v.2.0: expanded prediction of bacterial protein

subcellular localization and insights gained from comparative proteome analysis. Bioinformatics (Oxford, England) **21:**617-623.

22. **Groisman, E. A., and H. Ochman.** 1997. How *Salmonella* became a pathogen. Trends in microbiology **5:**343-349.

23. **Hansen-Wester, I., B. Stecher, and M. Hensel.** 2002. Type III secretion of *Salmonella enterica* serovar Typhimurium translocated effectors and SseFG. Infection and immunity **70:**1403-1409.

24. **Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galan.** 1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell **93:**815-826.

25. **Hensel, M.** 2004. Evolution of pathogenicity islands of *Salmonella enterica.* International Journal of Medical Microbiology **294:**95-102.

26. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden.** 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Molecular microbiology **30:**163-174.

27. **Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky.** 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proceedings of the National Academy of Sciences of the United States of America **96:**2396-2401.

28. **Hone, D. M., A. M. Harris, S. Chatfield, G. Dougan, and M. M. Levine.** 1991. Construction of genetically defined double *aro* mutants of *Salmonella typhi*. Vaccine **9:**810-816.

29. **Kaniga, K., M. S. Compton, R. Curtiss, 3rd, and P. Sundaram.** 1998. Molecular and functional characterization of *Salmonella enterica* serovar Typhimurium *poxA* gene: effect on attenuation of virulence and protection. Infection and immunity **66:**5599-5606.

30. **Kaniga, K., I. Delor, and G. Cornelis.** 1991. A wide host range suicide vector for improving reverse genetics in gram negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137-141.

31. **Knodler, L. A., J. Celli, W. D. Hardt, B. A. Vallance, C. Yip, and B. B. Finlay.** 2002. *Salmonella* effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems. Molecular microbiology **43:**1089-1103.

32. **Kuhle, V., and M. Hensel.** 2004. Cellular microbiology of intracellular *Salmonella enterica*: functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2. Cellular and Molecular Life Science **61:**2812-2826.

33. **Lai, X. H., I. Arencibia, A. Johansson, S. N. Wai, J. Oscarsson, S. Kalfas, K. G. Sundqvist, Y. Mizunoe, A. Sjostedt, and B. E. Uhlin.** 2000. Cytocidal and apoptotic effects of the ClyA protein from *Escherichia coli* on primary and cultured monocytes and macrophages. Infection and immunity **68:**4363-4367.

34. **Linehan, S. A., A. Rytkonen, X. J. Yu, M. Liu, and D. W. Holden.** 2005. SlyA Regulates Function of *Salmonella* Pathogenicity Island 2 (SPI-2) and Expression of SPI-2-Associated Genes. Infection and immunity **73:**4354-4362.

35. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-408.

36. **Lostroh, C. P., and C. A. Lee.** 2001. The *Salmonella* pathogenicity island-1 type III secretion system. Microbes and infection / Institut Pasteur **3:**1281-1291.

37. **Lucchini, S., G. Rowley, M. D. Goldberg, D. Hurd, M. Harrison, and J. C. Hinton.** 2006. H-NS mediates the silencing of laterally acquired genes in bacteria. PLoS pathogens **2:**e81.

38. **Ludwig, A., C. von Rhein, S. Bauer, C. Huttinger, and W. Goebel.** 2004. Molecular analysis of cytolysin A (ClyA) in pathogenic *Escherichia coli* strains. Journal of bacteriology **186:**5311-5320.

39. **Lundberg, U., U. Vinatzer, D. Berdnik, A. von Gabain, and M. Baccarini.** 1999. Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. Journal of bacteriology **181:**3433-3437.

40. **Marcus, S. L., J. H. Brumell, C. G. Pfeifer, and B. B. Finlay.** 2000. *Salmonella* pathogenicity islands: big virulence in small packages. Microbes and infection / Institut Pasteur **2:**145-156.

41. **McClelland, M., K. E. Sanderson, S. W. Clifton, P. Latreille, S. Porwollik, A. Sabo, R. Meyer, T. Bieri, P. Ozersky, M. McLellan, C. R. Harkins, C. Wang, C. Nguyen, A. Berghoff, G. Elliott, S. Kohlberg, C. Strong, F. Du, J. Carter, C. Kremizki, D. Layman, S. Leonard, H. Sun, L. Fulton, W. Nash, T. Miner, P. Minx, K. Delehaunty, C. Fronick, V. Magrini, M. Nhan, W. Warren, L. Florea, J. Spieth, and R. K. Wilson.** 2004. Comparison of genome

degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. Nature Genetics **36:**1268-1274.

42. **Monack, D. M., D. M. Bouley, and S. Falkow.** 2004. *Salmonella typhimurium* persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization. The Journal of experimental medicine **199:**231-241.

43. **Monack, D. M., A. Mueller, and S. Falkow.** 2004. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nature reviews **2:**747-765.

44. **Murray, G. L., S. R. Attridge, and R. Morona.** 2006. Altering the length of the lipopolysaccharide O antigen has an impact on the interaction of *Salmonella enterica* serovar Typhimurium with macrophages and complement. Journal of bacteriology **188:**2735-2739.

45. **O'Callaghan, D., and A. Charbit.** 1990. High efficiency transformation of *Salmonella typhimurium* and *Salmonella typhi* by electroporation. Molecular and General Genetics **223:**156-158.

46. **Ochman, H., and E. A. Groisman.** 1996. Distribution of pathogenicity islands in *Salmonella spp.* Infection and immunity **64:**5410-5412.

47. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299-304.

48. **Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman.** 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proceedings of the National Academy of Sciences of the United States of America **93:**7800-7804.

49. **Oscarsson, J., Y. Mizunoe, L. Li, X. H. Lai, A. Wieslander, and B. E. Uhlin.** 1999. Molecular analysis of the cytolytic protein ClyA (SheA) from *Escherichia coli*. Molecular microbiology **32:**1226-1238.

50. **Oscarsson, J., M. Westermark, S. Lofdahl, B. Olsen, H. Palmgren, Y. Mizunoe, S. N. Wai, and B. E. Uhlin.** 2002. Characterization of a pore-forming cytotoxin expressed by *Salmonella enterica* serovars Typhi and Paratyphi A. Infection and immunity **70:**5759-5769.

51. **Pan, Q., X. L. Zhang, H. Y. Wu, P. W. He, F. Wang, M. S. Zhang, J. M. Hu, B. Xia, and J. Wu.** 2005. Aptamers that preferentially bind type IVB pili and inhibit human monocytic-cell invasion by *Salmonella enterica* serovar Typhi. Antimicrobial agents and chemotherapy **49:**4052-4060.

52. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848-852.

53. **Parry, C. M., T. T. Hien, G. Dougan, N. J. White, and J. J. Farrar.** 2002. Typhoid fever. The New England journal of medicine **347:**1770-1782.

54. **Parsons, D. A., and F. Heffron.** 2005. *sciS*, an *icmF* homolog in *Salmonella enterica* serovar Typhimurium, limits intracellular replication and decreases virulence. Infection and immunity **73:**4338-4345.

55. **Paulin, S. M., A. Jagannathan, J. Campbell, T. S. Wallis, and M. P. Stevens.** 2007. Net replication of *Salmonella enterica* serovars Typhimurium and Choleraesuis in porcine intestinal mucosa and nodes is associated with their differential virulence. Infection and immunity **75:**3950-3960.

56. **Porwollik, S., E. F. Boyd, C. Choy, P. Cheng, L. Florea, E. Proctor, and M. McClelland.** 2004. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. Journal of bacteriology **186:**5883-5898.

57. **Raffatellu, M., D. Chessa, R. P. Wilson, R. Dusold, S. Rubino, and A. J. Baumler.** 2005. The Vi capsular antigen of *Salmonella enterica* serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. Infection and immunity **73:**3367-3374.

58. **Raffatellu, M., D. Chessa, R. P. Wilson, C. Tukel, M. Akcelik, and A. J. Baumler.** 2006. Capsule-mediated immune evasion: a new hypothesis explaining aspects of typhoid fever pathogenesis. Infection and immunity **74:**19-27.

59. **Raffatellu, M., R. L. Santos, D. Chessa, R. P. Wilson, S. Winter, C. A. Rossetti, S. D. Lawhon, H. Chu, T. Lau, C. L. Bevins, L. G. Adams, and A. J. Baumler.** 2007. The Capsule encoding *viaB* locus reduces IL-17 expression and mucosal innate responses in the bovine intestinal mucosa during infection with *Salmonella enterica* serotype Typhi. Infection and immunity.

60. **Richter-Dahlfors, A., A. M. Buchan, and B. B. Finlay.** 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. The Journal of experimental medicine **186:**569-580.

61. **Rosenshine, I., S. Ruschkowski, and B. B. Finlay.** 1994. Inhibitors of cytoskeletal function and signal transduction to study bacterial invasion. Methods in enzymology **236:**467-476.

62. **Roy, D., D. R. Liston, V. J. Idone, A. Di, D. J. Nelson, C. Pujol, J. B. Bliska, S. Chakrabarti, and N. W. Andrews.** 2004. A process for controlling intracellular bacterial infections induced by membrane injury. Science **304:**1515-1518.

63. **Rytkonen, A., J. Poh, J. Garmendia, C. Boyle, A. Thompson, M. Liu, P. Freemont, J. C. Hinton, and D. W. Holden.** 2007. SseL, a *Salmonella* deubiquitinase required for macrophage killing and virulence. Proceedings of the National Academy of Sciences of the United States of America **104:**3502-3507.

64. **Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush.** 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques **34:**374-378.

65. **Santos, R. L., S. Zhang, R. M. Tsolis, R. A. Kingsley, L. G. Adams, and A. J. Baumler.** 2001. Animal models of *Salmonella* infections: enteritis versus typhoid fever. Microbes and infection / Institut Pasteur **3:**1335-1344.

66. **Schwan, W. R., X. Z. Huang, L. Hu, and D. J. Kopecko.** 2000. Differential bacterial survival, replication, and apoptosis-inducing ability of *Salmonella* serovars within human and murine macrophages. Infection and immunity **68:**1005-1013.

67. **Soderblom, T., C. Oxhamre, S. N. Wai, P. Uhlen, A. Aperia, B. E. Uhlin, and A. Richter-Dahlfors.** 2005. Effects of the *Escherichia coli* toxin cytolysin A on mucosal immunostimulation via epithelial Ca2+ signalling and Toll-like receptor 4. Cellular microbiology **7:**779-788.

68. **Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold, and W. D. Hardt.** 2000. Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Molecular microbiology **36:**1206-1221.

69. **Sukhan, A., T. Kubori, J. Wilson, and J. E. Galan.** 2001. Genetic analysis of assembly of the *Salmonella enterica* serovar Typhimurium type III secretion-associated needle complex. Journal of bacteriology **183:**1159-1167.

70. **Tierrez, A., and F. Garcia-del Portillo.** 2005. New concepts in *Salmonella* virulence: the importance of reducing the intracellular growth rate in the host. Cellular microbiology **7:**901-909.

71. **Tzokov, S. B., N. R. Wyborn, T. J. Stillman, S. Jamieson, N. Czudnochowski, P. J. Artymiuk, J. Green, and P. A. Bullough.** 2006. Structure of the hemolysin E (HlyE, ClyA, and SheA) channel in its membrane-bound form. The Journal of biological chemistry **281:**23042-23049.

72. **Uzzau, S., N. Figueroa-Bossi, S. Rubino, and L. Bossi.** 2001. Epitope tagging of chromosomal genes in *Salmonella*. Proceedings of the National Academy of Sciences of the United States of America **98:**15264-15269.

73. **Vernikos, G. S., N. R. Thomson, and J. Parkhill.** 2007. Genetic flux over time in the *Salmonella* lineage. Genome Biol **8:**R100.

74. **von Rhein, C., K. P. Hunfeld, and A. Ludwig.** 2006. Serologic evidence for effective production of cytolysin A in *Salmonella enterica* serovars Typhi and Paratyphi A during human infection. Infection and immunity **74:**6505-6508.

75. **Wai, S. N., B. Lindmark, T. Soderblom, A. Takade, M. Westermark, J. Oscarsson, J. Jass, A. Richter-Dahlfors, Y. Mizunoe, and B. E. Uhlin.** 2003. Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. Cell **115:**25-35.

76. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-199.

77. **Waterman, S. R., and D. W. Holden.** 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cellular microbiology **5:**501-511.

78. **Worley, M. J., K. H. Ching, and F. Heffron.** 2000. *Salmonella* SsrB activates a global regulon of horizontally acquired genes. Molecular microbiology **36:**749-761.

FIGURE LEGENDS

Figure 1. *taiA* (*STY1499*) and *clyA* are arranged as an operon and are induced during infection of human macrophages. A) DNA region encoding *taiA* and *clyA* in Typhi and the corresponding DNA region in Typhimurium. B) The transcription unit was analysed by reverse transcriptase (RT) -PCR. Primers used are depicted in A: 1) 1498-R, 2) 1498-F, 3) 1499-R and 4) 1499-F. NRT, no reverse transcriptase (negative control). C) qPCR validation of expression of *taiA* and *clyA* inside human macrophages, compared to the supernatant of infection.

Figure 2. ClyA reduced survival of Typhi inside human macrophages and was cytotoxic for epithelial cells. THP-1 cells and HeLa cells were infected with wild-type (control) and DEF062 (Δ*clyA*) strain or the complemented strain DEF123 (wild-type + pWSKclyA ; control) and DEF124 (Δ*clyA* + pWSKclyA). For THP-1 cells, the phagocytosis rate and survival rate at 48 h post-infection are shown and for HeLa cells the invasion rate is shown (A). The cytotoxicity toward THP-1 and HeLa cells was investigated by quantifying LDH release (B). Results are shown as a percentage of the control value for each replicate. Experiments were replicated at least 3 times independently, * $P < 0.05$.

Figure 3. TaiA is involved in early interaction with host cells. THP-1 cells or HeLa cells were infected with the wild-type (control), DEF061 (Δ*taiA*) strain or the complemented strains DEF074 (wild-type + pWSK*taiA*) and DEF075 (Δ*taiA* + pWSK*taiA*). For THP-1 cells, two phenotypes were investigated: the phagocytic rate and the survival rate at 48 h

(A). The phagocytic rate for DEF061 compared to the wild-type was also investigated in the presence of cytochalasin D (B). For HeLa cells the invasion rate was investigated (C). See text for details. Results are shown as a percentage of the control value for each replicate. Experiments were replicated at least 3 times independently, * $P < 0.05$.

Figure 4. Production of TaiA in SPI-1 inducing media. Bacteria expressing TaiA with a double HA-tag at its C-terminal was used to investigate TaiA secretion. Bacteria harboring the vector alone were used as a negative control. Bacteria were grown in LB-NaCl O/N standing (SPI-1 inducing condition). Proteins in the culture supernatant (S) and in the bacterial pellet (P) were subjected to western blotting.

Figure 5. Production of TaiA in SPI-2 inducing media. A) Bacteria were grown in LPM pH 5.8 broth aerobically (SPI-2 inducing condition) and the protein in supernatant (S) and bacterial pellet (P) were harvested and subjected to western blotting. See text for details. B) Real-Time qPCR was used to compare the expression of *taiA* and *clyA* in the wild-type strain and in a Δ*ssrB* strain at 24h post-infection of human macrophages. The fold change represents the level of normalized expression in the Δ*ssrB* strain compared to the wild-type strain. *sseB* was used as a control for SsrB dependant expression. See material and methods for details.

Figure 6. *E. coli* harboring TaiA is more invasive. HeLa cells were infected with *E. coli* harboring pWSK29 (control) and *E. coli* harboring pWSK*taiA*. Adherence rate and invasion rate are shown. Results are shown as a percentage of the control value for each replicate. Experiments were replicated at least 3 times independently, * $P < 0.05$.

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

Strain or plasmid	Characteristics	Reference or source
Typhi		
ISP1820	Wild-type Typhi	(28)
DEF053	ISP1820 (pWSK29)	This study
DEF061	ISP1820 Δ*taiA*	This study
DEF062	ISP1820 Δ*clyA*	This study
DEF074	ISP1820 (pSIF029)	This study
DEF075	DEF061 (pSIF029)	This study
DEF123	ISP1820 (pSIF051)	This study
DEF124	DEF062 (pSIF051)	This study
DEF131	DEF061 (pWSK29)	
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
E. coli		
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*	(29)
Plasmid		
pMEG-375	*sacRB mobRP4 ori*R6K. Cm^r, Ap^r	(13)
pWSK29	Low-copy-number cloning vector, Amp^r	(76)
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 2 HA 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

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	CGGGATCCCTATTAAGCGTAGTCTGGGACGTCGTATGGG TAAAGAGCGTAGTCTGGGACGTCGTATGGGTAAGATCTA CGCAGGGTACGATTACTC
16s-F	CGGGGAGGAAGGTGTTGTG
16s-R	CAGCCCGGGGATTTCACATC
1499-F	ATATCACCGATGCGGTGGGAATC
1499-R	ACTTTCACCATTCCATCTTCCGGC
1498-F	ACGGAAACCGAAACAACCAGATTC
1498-R	GCGTCTTCTTACCGTGTCTTTGTTGG
sseB-F	ATATGGCGATCATGGGAAGCTGGA
sseB-R	TCGGTATTCCGGTTGGCGTCATTA

FIGURES

Figure 1.

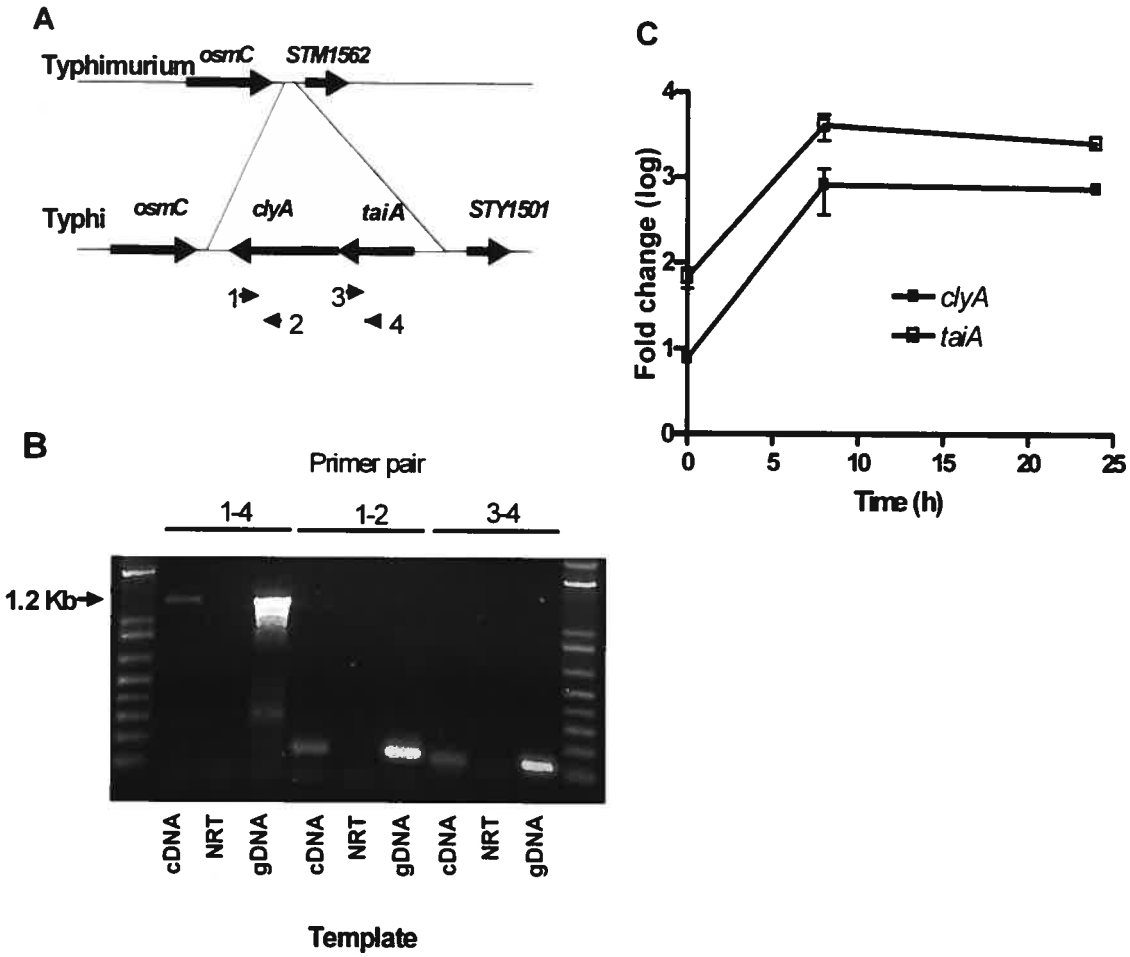


Figure 2.

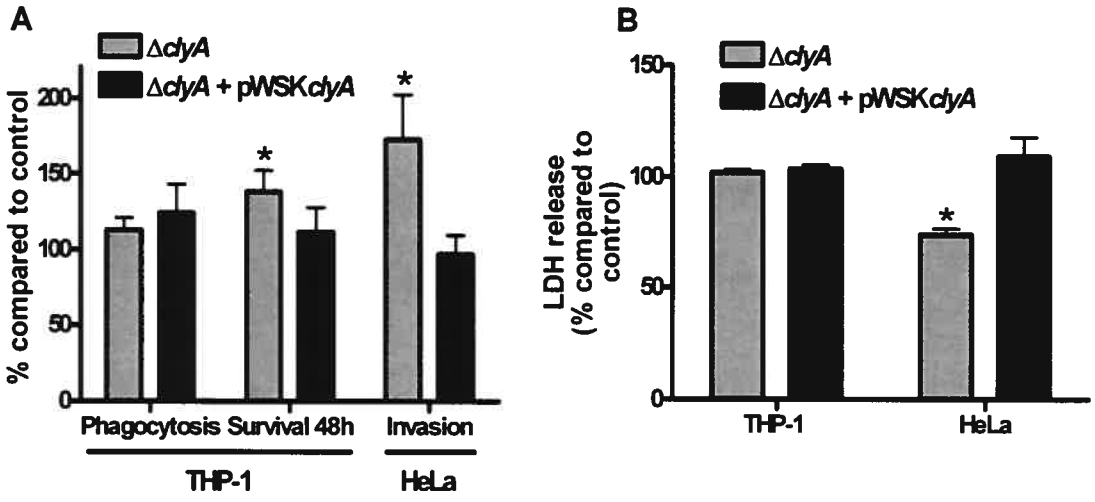

A
ΔclyA
ΔclyA + pWSKclyA
% compared to control
200
150
100
50
0
*
*
Phagocytosis Survival 48h
Invasion
THP-1
HeLa
B
ΔclyA
ΔclyA + pWSKclyA
LDH release (% compared to control)
150
100
50
0
*
THP-1
HeLa

Figure 3.

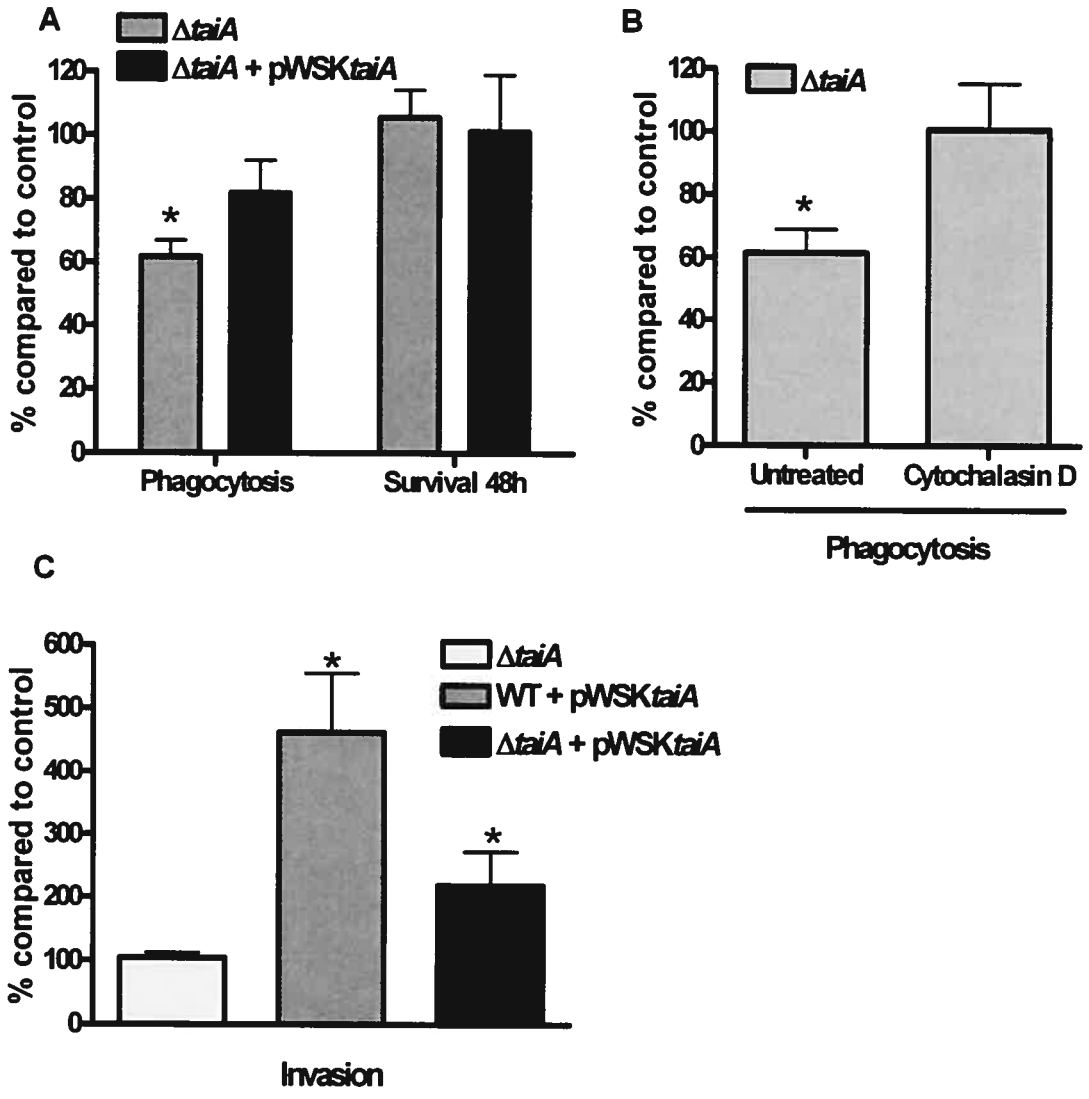

A
ΔtaiA
ΔtaiA + pWSKtaiA
% compared to control
120
100
80
60
40
20
0
*
Phagocytosis
Survival 48h
B
ΔtaiA
% compared to control
120
100
80
60
40
20
0
*
Untreated
Cytochalasin D
Phagocytosis
C
ΔtaiA
WT + pWSKtaiA
ΔtaiA + pWSKtaiA
% compared to control
600
500
400
300
200
100
0
*
*
Invasion

Figure 4.

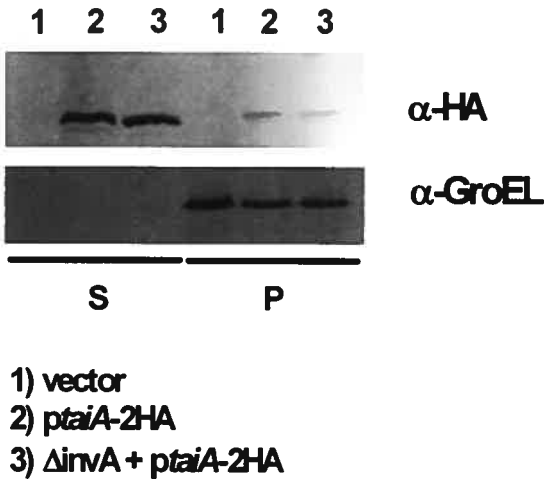


Figure 5.

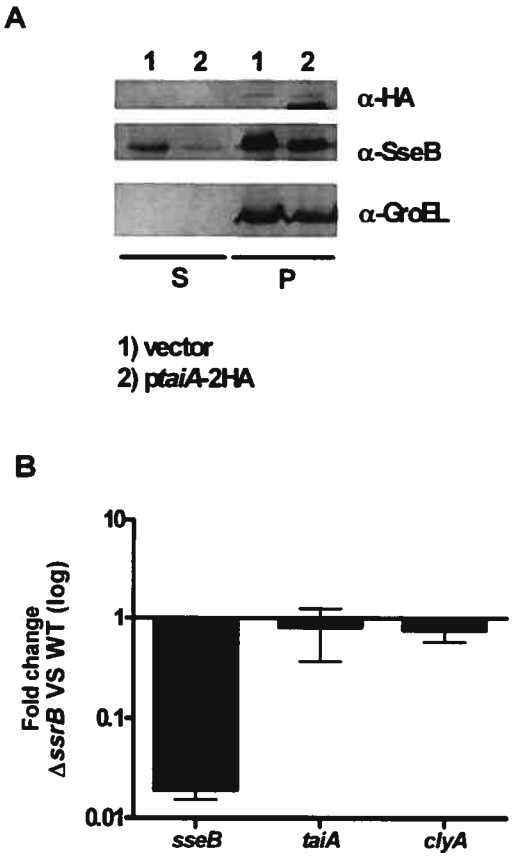
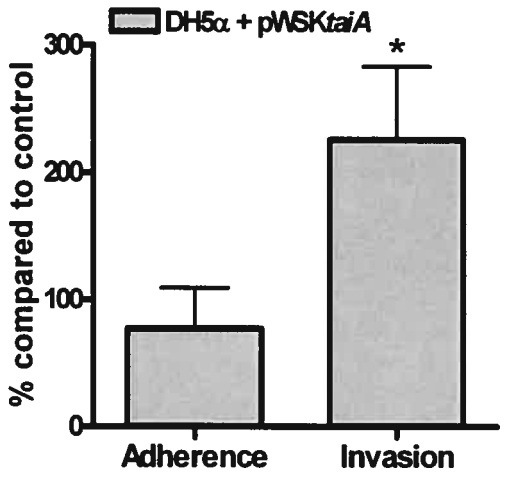


Figure 6.


DH5α + pWSK*taiA*
300
200
100
0
% compared to control
*
Adherence
Invasion

CHAPITRE 6: Discussion

Typhi est un des rares sérovars de *S. enterica* capable de causer une infection systémique chez l'homme. Une telle capacité, au niveau de la pathogenèse, indique que les mécanismes de virulence utilisés comportent des spécificités qui lui sont propres. Bien que le modèle murin de la fièvre typhoïde ait permis d'identifier plusieurs mécanismes de virulence utilisés par *Salmonella* pour causer une infection systémique, ce modèle ne permet pas d'identifier les mécanismes spécifiques à Typhi. Les bactéries réagissent à leur environnement en adaptant leur profil d'expression génique (38). Ceci est illustré par le nombre important de régulateurs associés à la virulence et par le fait que leur délétion diminue la virulence (41). Les gènes exprimés par les bactéries pathogènes *in vivo* représentent des facteurs qui leur confèrent un avantage dans cet environnement. Il est donc nécessaire, pour mieux comprendre la pathogenèse de Typhi, d'identifier et de caractériser les facteurs de virulence propres à Typhi qui sont exprimés dans des conditions d'infection.

Plusieurs approches ont été développées afin d'identifier les gènes exprimés par les bactéries pathogènes dans des conditions d'infections. Par exemple, les méthodes de IVET (141, 142) et des biopuces (58) ont été utilisées pour identifier les mécanismes de virulence de Typhimurium. La méthode SCOTS a été utilisée avec succès pour étudier l'expression génique chez plusieurs bactéries pathogènes comme *Mycobacterium* (80, 108), *Salmonella* (44, 155), *E. coli* pathogène aviaire (54), *Actinobacillus* (8, 9) et *Helicobacter pylori* (81). L'avantage de cette méthode est qu'elle ne nécessite pas de

modification génétique de la bactérie étudiée ou du modèle d'infection utilisé habituellement. Pour ces raisons, nous avons décidé d'appliquer cette méthode pour l'étude des gènes exprimés par Typhi pendant l'infection des macrophages humains. Dans un premier temps, la technique SCOTS a été utilisée pour capturer les gènes de Typhi exprimés dans les macrophages humains avec une étape d'enrichissement pour capturer les gènes absents du génome de Typhimurium. Pour des raisons de simplicité, cette approche est appelée SCOTS. Dans un deuxième temps, SCOTS a été utilisée conjointement avec les biopuces pour obtenir le profil d'expression global de Typhi pendant l'infection de macrophages humains. Cette approche est appelée biopuces. L'utilisation de ces approches s'est révélée fructueuse puisque trois facteurs de virulence spécifiques à Typhi, ClyA, TaiA et le fimbriae Stg, ont été identifiés et caractérisés.

6.1. L'approche de SCOTS

Le génome de Typhi a été séquencé et comparé à celui de Typhimurium pour identifier les gènes présents uniquement chez Typhi (146, 167). Cependant, cette approche bioinformatique ne permet pas d'obtenir de l'information sur l'expression *in vivo* de ces gènes. L'utilisation de SCOTS avec une étape d'enrichissement a permis d'identifier 36 gènes de Typhi, exprimés dans les macrophages à 2 h post-infection, mais absents du génome de Typhimurium (Chapitre 2, Table 1). Ces 36 gènes appartiennent à 15 régions génomiques absentes chez Typhimurium. Certaines de ces régions sont des SPIs et des prophages, qui sont impliqués dans le transfert horizontal de gènes et dans l'évolution des bactéries (83, 204). Certains des gènes identifiés sont présents aussi chez Paratyphi, qui est aussi capable de causer une infection systémique chez l'homme. Cette

observation vient appuyer l'hypothèse que ces gènes soient impliqués dans la capacité de Typhi d'infecter l'homme de façon systémique.

Certains des gènes capturés sont des facteurs de virulence déjà connus. Par exemple, les gènes, *tviE* et *vexB*, impliqués dans la synthèse et le transport de la capsule Vi, ont été capturés. La capsule Vi est impliquée dans la survie à l'intérieur des macrophages humains et dans la réduction de la production de TNF-α (102). La capture des gènes *tviE* et *vexB* démontre que la méthode permet d'identifier des gènes possiblement impliqués dans la virulence de Typhi. Des 36 gènes identifiés, 23 codent pour des protéines hypothétiques ou des protéines qui possèdent une fonction présumée selon leur homologie.

L'approche de SCOTS a permis d'identifier le gène *stgC*, codant pour le placier de l'opéron fimbriaire *stg*, ce qui suggérait que ce fimbriae était impliqué dans l'interaction de Typhi avec les cellules humaines. Par contre, le placier du fimbriae Stg contient un codon d'arrêt interne et est classé comme pseudogène. Nous avons démontré que ce fimbriae est tout de même fonctionnel et qu'il est impliqué dans la virulence de Typhi. Le fimbriae Stg semble contribuer à l'adhésion de Typhi aux cellules épithéliales (Chapitre 3, Fig. 3). Par contre, ce fimbriae réduit la phagocytose de Typhi par les macrophages (Chapitre 3, Fig. 3). Ceci montre que les résultats obtenus par SCOTS sont valables et qu'un nouveau facteur de virulence a pu être identifié par cette approche.

6.2. L'approche des biopuces

SCOTS ne permet pas d'identifier globalement tous les gènes exprimés par Typhi pendant l'infection, contrairement à la technique des biopuces. Par contre, l'utilisation des biopuces pose des problèmes pour obtenir le transcriptome d'une bactérie pathogène dans un contexte d'infection, à cause de la quantité et de la pureté des transcrits bactériens disponibles, comme expliqué à la section 1.6.4. Ceci est bien illustré par l'image de la biopuce hybridée avec l'ADNc non traité par SCOTS, puisque très peu de gènes sont détectés et que le signal est très faible (Chapitre 4, Fig. 1). Pour contourner ces obstacles, les transcrits obtenus de chacune des conditions à l'étude ont été purifiés et amplifiés par SCOTS, marqués avec des fluorochromes et hybridés aux biopuces de *Salmonella* (62). Pour valider l'utilisation conjointe de SCOTS et des biopuces, le profil d'expression de 8 gènes, choisis au hasard, a été confirmé par PCR quantitatif en temps réel (qPCR). Les résultats obtenus par biopuces corrèlent bien avec ceux obtenus par qPCR ($r = 0.79$). De plus, le profil d'expression du SST3-1 et celui du SST3-2 concorde avec leur fonction biologique spécifique. Le SST3-1, nécessaire à l'invasion, est réprimé à l'intérieur des macrophages et le SST3-2, impliqué dans la survie, est induit (Chapitre 4, Fig. 2). Il semble donc que SCOTS et les biopuces peuvent être utilisés avec succès pour obtenir le transcriptome de Typhi dans des conditions qui ne permettaient pas d'appliquer directement la méthode des biopuces. L'utilisation conjointe de SCOTS et des biopuces devrait permettre de faciliter l'obtention de transcriptomes d'autres bactéries pathogènes dans des conditions d'infection. Les résultats contenus dans le chapitre 4 montrent que SCOTS est capable de purifier et d'amplifier correctement les transcrits bactériens. En ce sens, il a été suggéré que cette

méthode soit utilisée pour l'étude du transcriptome de *Mycobacterium tuberculosis* pendant l'infection (123).

L'obtention du transcriptome de Typhi dans des conditions d'infection est une contribution majeure aux connaissances de sa pathogenèse, puisque c'est la première fois que ce genre d'étude est effectué sur ce pathogène. Plusieurs facteurs de virulence sont induits dans les macrophages comme le SST3-2 et les gènes de résistances aux peptides antimicrobiens (PA). Plusieurs gènes sont aussi réprimés comme les systèmes de transport du fer, qui sont aussi réprimés chez Typhimurium pendant l'infection de macrophages murins (58). Ces résultats supportent l'idée que le fer est facilement accessible dans la vacuole du macrophage, ce qui pourrait expliquer que plusieurs gènes impliqués dans le transport du fer sont des pseudogènes chez Typhi (167). Typhi semble aussi réprimer les systèmes impliqués dans la mobilité (flagelle et chemotaxis) (Fig. 2), ce qui est aussi le cas de Typhimurium (58). Il n'est évidement pas nécessaire de produire des flagelles lorsque la bactérie se retrouve dans un espace aussi restreint que le phagosome, d'autant plus que la flagelline (unité majeure du flagelle) est reconnue comme étant très immunogène (186). Ces résultats étaient attendus et confirment la validité de la méthode employée.

Au chapitre 4, le transcriptome de Typhi dans les macrophages humains a été comparé exhaustivement à celui de Typhimurium dans les macrophages murins (58). Il est possible que les écarts entre ces deux transcriptomes soient liés aux conditions expérimentales différentes, comme il a été discuté. La plus surprenante différence est l'induction beaucoup plus marquée par Typhi de gènes impliqués dans la réponse aux

PA, incluant le régulateur *phoP* et les gènes *pmrEF* et *pagP* (Chapitre 4 Fig. 2). Ceux-ci sont impliqués dans l'addition de groupement 4-aminoarabinose ou phosphoéthanolamine au phosphate du lipide A (229) et à l'ajout d'un groupement palmitate au lipide A (89). Il a d'ailleurs été démontré que les LPS récoltés de Typhimurium pendant l'infection de macrophages présentaient de telles modifications (78). Cela montre que le transcriptome de Typhi dans les macrophages est représentatif des changements d'expression reliés aux effets biologiques impliqués dans l'adaptation aux conditions prévalentes pendant l'infection. La condition contrôle utilisée semble être appropriée pour identifier les gènes induits à l'intérieur des macrophages par Typhi. Dans le cas du transcriptome de Typhimurium, la condition contrôle utilisée semble camoufler l'expression des gènes impliqués dans la résistance aux PA, peut être parce qu'ils sont déjà exprimés dans celle-ci.

Il a été proposé que Typhimurium utilise le gluconate comme source de carbone à l'intérieur de macrophages (58). Par contre, l'opéron *dgo* nécessaire à l'utilisation du gluconate est absent chez Typhi et les gènes *gntT* et *gntU* codant pour les transporteurs sont réprimés dans les macrophages (62). L'utilisation des acides gras comme source de carbone via le cycle du glyoxylate semble être importante pour la persistance de *M. tuberculosis* dans les macrophages humains et de Typhimurium dans le modèle murin (61, 147). Les gènes *aceA* et *aceB,* codant pour les enzymes du cycle du glyoxylate, sont induits à 8 h et 24 h et à 24 h respectivement. Les enzymes nécessaires à la dégradation des acides gras sont généralement produites dans les macrophages, tout au long de l'infection (33). Par contre, le transporteur de longues chaînes d'acide gras (plus grandes que 12 carbones), encodé par *fadL*, est réprimé dans les macrophages. Il semble

donc que Typhi utilise les acides gras courts (plus petit que 12 carbones) comme source de carbone à l'intérieur des macrophages.

Le transcriptome de Typhi dans les macrophages a aussi permis d'identifier 19 gènes absents chez Typhimurium et exprimés par Typhi à l'intérieur des macrophages, comparés au surnageant d'infection. Aucun de ces gènes n'avait été identifié avec la première approche. L'un d'eux, *cdtB* code pour une toxine impliquée dans la mort des cellules intestinales infectées par Typhi (91). Nos résultats laissent présager que CdtB a aussi cet effet dans les macrophages, puisqu'il est induit pendant leur infection. Les gènes *clyA* et *taiA* (STY1499) sont très fortement exprimés tout au long de l'infection, dès le temps 0, et sont situés sur le même locus d'ADN. L'implication des gènes *taiA* et *clyA* dans la pathogenèse de Typhi a été évaluée. TaiA semble augmenter le taux de phagocytose de Typhi par les macrophages (Chapitre 5, Fig. 3). TaiA est une nouvelle protéine sécrétée par Typhi indépendamment du SST3-1 et du SST3-2 (Chapitre 5, Fig. 4 et 5). ClyA est cytotoxique pour les cellules épithéliales (Chapitre 5, Fig 2). Par contre, ClyA n'est pas cytotoxique pour les macrophages, mais elle réduit le taux de survie de Typhi dans ces cellules (Chapitre 5, Fig 2). Cette activité pourrait permettre de contrôler la croissance de Typhi dans les macrophages, ce qui a été relié à la capacité de *Salmonella* d'établir une infection systémique persistante, comme la typhoïde (153, 205). Ces deux protéines semblent avoir des fonctions complémentaires pour permettre d'utiliser les macrophages comme réservoir.

Le fimbriae *stg* et *taiA* ont des effets opposés sur l'interaction de Typhi avec les macrophages humains. Le fimbriae *stg* semble diminuer la phagocytose tandis que *taiA*

semble l'augmenter. Cette activité antagoniste peut paraître absurde, d'un point de vue évolutif, mais reflète possiblement que la phagocytose doit être contrôlée précisément pour optimiser l'entrée de Typhi dans les macrophages et permettre l'établissement d'une infection systémique.

6.3. Opportunité de recherche

Les autres gènes uniques que nous avons identifiés peuvent aussi être impliqués dans la pathogenèse de Typhi, comme il a été démontré pour *stg*, *clyA* et *taiA*. Par exemple, la méthode de SCOTS a identifié 2 gènes, *STY3948* et *STY3950*, situés dans la même région génomique (Chapitre 2, Table 1). Cette région code aussi pour le gène *STY3949* qui s'avère aussi absent du génome de Typhimurium. Cette région est présente seulement chez les sérovars Typhi, Agona, Sendai et Senftenberg et chez *S. bongori*. *STY3948* et *STY3950* semblent moins exprimés dans les macrophages murins que dans les macrophages humains (Chapitre 2, Fig. 2 et Annexe A2). Ces gènes pourraient être impliqués dans la spécificité d'hôte de Typhi s'ils s'avèrent être importants pour sa survie dans les macrophages humains. De plus, STY3948 et STY3949 semblent être des protéines de la membrane externe selon l'analyse bio-informatique effectuée sur le site Tigr CMR (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). Les protéines de la membrane externe peuvent interagir avec la cellule hôte ou avec les mécanismes de défense de l'hôte. Par exemple, PgtE est une protéine située dans la membrane externe et est impliquée dans la dégradation des peptides antimicrobiens (84). Il serait donc intéressant d'évaluer l'implication de cette région dans la pathogenèse de Typhi.

La caractérisation de l'opéron fimbriaire *stg* a permis de démontrer que ce fimbriae est fonctionnel, même si le placier est un pseudogène (Chapitre 3). Cette déficience peut être complémentée par le placier d'un autre opéron fimbriaire, ce qui expliquerait que le fimbriae Stg soit quand même fonctionnel. L'effet du placier pseudogène pourrait être investigué en remplaçant son codon stop interne par le codon de la tyrosine retrouvé au même endroit chez le fimbriae *stg* de *S. bongori*. Le niveau de sécrétion de la sous-unité majeure StgA pourrait être comparé dans cette souche et dans la souche sauvage. De plus, le mécanisme par lequel le fimbriae Stg réduit la phagocytose est encore inconnu. Il est possible que cet effet soit une conséquence du camouflage d'une structure de surface de Typhi par le fimbriae ou de la liaison de StgD, l'adhésine du fimbriae, à un récepteur du macrophage. Cette possibilité pourrait être investiguée en utilisant la méthode de « phage display » pour identifier des ligands potentiels de StgD (156).

Le génome de Typhi code pour plusieurs autres fimbriae (207). Selon nos données de biopuces, la majorité des opérons fimbriaires semblent être réprimés à l'intérieur des macrophages sauf pour l'opéron *saf* qui est induit immédiatement après la phagocytose (62). Typhimurium semble aussi induire *saf* à l'intérieur des macrophages (58) mais la sous-unité *safA* de Typhi possède seulement 44% d'identité avec celle de Typhimurium, ce qui pourrait être le résultat d'une variation allélique (65). De plus, *safD*, qui code pour l'adhésine, a 22,8% d'identité avec *sefD* de Enteritidis qui semble être important lors de son interaction avec les macrophages (56). À la lumière de ces constatations, le fimbriae *saf* pourrait avoir un rôle dans l'interaction de Typhi avec les macrophages humains, d'autant plus que chez Typhi, *sefA* et *sefD* sont des pseudogènes

(207). Il serait utile de déterminer son rôle dans l'interaction de Typhi avec les macrophages humains.

L'étude de l'implication de ClyA dans la pathogenèse de Typhi a soulevé quelques questions (Chapitre 5). Le mécanisme par lequel ClyA diminue la survie de Typhi dans les macrophages est encore inconnu. Il pourrait être utile de clarifier en premier lieu l'emplacement de ClyA dans les macrophages par microscopie à fluorescence ou électronique. Le transport de ClyA est assuré par des OMV qui pourraient fusionner à la membrane de la vacuole, bien que des évidences directes n'aient pas été présentées à ce jour (120). Par contre, il a été démontré que pendant l'infection de cellules épithéliales par Typhimurium, du LPS s'accumulait dans la membrane de la SCV et que des vésicules contenant du LPS bourgeonnait de la membrane de la SCV (73). L'incorporation de LPS dans la membrane de la SCV suite à la fusion de OMV pourrait expliquer ce phénomène. La fusion d'OMV avec la SCV pourrait permettre l'insertion de pores formés de ClyA dans la membrane de la vacuole qui permettrait un échange de molécules avec le cytoplasme ou même la lyse de la vacuole, lorsque la quantité d'OMV produite atteint un certain seuil. *Salmonella* n'est pas capable de survivre dans le cytoplasme de la cellule (17, 169) et la lyse de la vacuole par ClyA réduirait donc la survie de Typhi dans les macrophages. La quantité de ClyA produite est dépendante de la quantité de bactéries présentent dans la vacuole et pourrait être un mécanisme pour contrôler la croissance de Typhi. Outre la lyse de la vacuole, ClyA pourrait aussi affecter les bactéries présentent dans la vacuole. En effet, les OMV sont capables de fusionner à la membrane externe des bactéries et de délivrer leur contenu dans le périplasme (144). Par exemple, les OMV de *Pseudomonas aeruginosa*

transportent des protéines qui lysent les autres genres bactériens (131). Si les OMV de Typhi ne fusionnent pas à la membrane de la vacuole, leur accumulation dans la SCV pourrait les mener à fusionner avec les membranes bactériennes. Ceci aurait pour effet d'insérer ClyA dans la membrane externe des bactéries, ce qui pourrait avoir un effet délétère sur la viabilité ou sur les activités de Typhi dans la vacuole.

TaiA semble être une nouvelle protéine sécrétée qui augmente le niveau de phagocytose des macrophages. Plusieurs points restent en suspend. Le mécanisme par lequel TaiA augmente la phagocytose de Typhi n'est pas encore connu. Il est possible que cet effet soit dépendant de l'interaction de TaiA avec un récepteur à la surface du macrophage. L'invasine de *Yersinia enterolitica* induit l'internalisation de la bactérie en se liant à l'intégrine β1 à la surface de la cellule hôte (223). L'approche du « phage display » suggéré pour le fimbriae Stg serait aussi applicable dans ce cas pour trouver un récepteur potentiel de TaiA (156). La sécrétion de TaiA ne semble pas être dépendante des SST3 et elle est beaucoup trop petite pour contenir les domaines nécessaires à son autotransport (118). Le mode de sécrétion de TaiA reste donc à élucider. Puisque TaiA et ClyA sont co-transcrit, co-exprimés et sont toutes deux sécrétés, il est envisageable que TaiA soit sécrétée et transportée par les OMV comme ClyA. Bien que leurs fonctions soient différentes, il est aussi possible qu'elles interagissent ensemble à un moment donné pendant leur transport, ce qui pourrait être étudié par co-immunoprécipitation.

CHAPITRE 7 : Conclusion

Il est difficile d'étudier les mécanismes de virulence d'une bactérie pathogène restreinte à l'homme comme Typhi puisqu'il n'existe pas de modèle animal. Plusieurs groupes de recherches ont utilisé Typhimurium dans le modèle murin de la fièvre typhoïde. Par contre, seul Typhi est capable d'infecter l'homme de façon systémique, ce qui indique que Typhi possède et utilise des facteurs de virulence qui lui sont propres. L'approche de SCOTS et celle des biopuces ont permis d'identifier des gènes uniques à Typhi (absent du génome de Typhimurium) exprimés dans les macrophages humains. Certains des gènes uniques identifiés ont été caractérisés. Nous avons démontré que ClyA, TaiA et le fimbriae Stg sont des nouveaux facteurs de virulence propres à Typhi et impliqués dans sa pathogenèse. Les méthodes utilisées sont complémentaires puisqu'elles ont permis d'identifier des gènes uniques différents. Par exemple, le fimbriae *stg* n'est pas induit *in vivo*, comparativement au surnageant d'infection, selon la méthode des biopuces. Ceci peut être expliqué par l'expression de *stg* dans la condition contrôle, qui masque son expression *in vivo*. De plus, la méthode de SCOTS n'a pas identifié les gènes *clyA* et *taiA*, probablement parce que cette méthode n'est pas exhaustive. De plus, le transcriptome de Typhi dans les macrophages permet de générer plusieurs hypothèses quant à sa pathogenèse. Cette thèse a donc permis de parfaire les connaissances sur la pathogenèse de Typhi. L'utilisation de SCOTS pour purifier et amplifier les transcrits bactériens semble être un bon moyen de parvenir à utiliser les biopuces pour étudier le transcriptome de bactéries pathogènes dans un contexte d'infection. Cette méthode devrait permettre d'étudier plusieurs autres pathogènes dans

des conditions ou la quantité de transcrits bactériens est faibles. Il s'agit d'une avancée majeure dans le domaine de la transcriptomique bactérienne.

Bibliographie

1. **Alix, E., and A. B. Blanc-Potard.** 2007. MgtC: a key player in intramacrophage survival. Trends in microbiology **15:**252-256.
2. **Alksne, L. E., and S. J. Projan.** 2000. Bacterial virulence as a target for antimicrobial chemotherapy. Current opinion in biotechnology **11:**625-636.
3. **Allard, M., H. Moisan, E. Brouillette, A. L. Gervais, M. Jacques, P. Lacasse, M. S. Diarra, and F. Malouin.** 2006. Transcriptional modulation of some *Staphylococcus aureus* iron-regulated genes during growth in vitro and in a tissue cage model in vivo. Microbes and infection / Institut Pasteur **8:**1679-1690.
4. **Anderson, D. M., and O. Schneewind.** 1997. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. Science **278:**1140-1143.
5. **Atkins, H. S., M. Morton, K. F. Griffin, M. G. Stokes, J. P. Nataro, and R. W. Titball.** 2006. Recombinant *Salmonella* vaccines for biodefence. Vaccine **24:**2710-2717.
6. **Bader, M. W., S. Sanowar, M. E. Daley, A. R. Schneider, U. Cho, W. Xu, R. E. Klevit, H. Le Moual, and S. I. Miller.** 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell **122:**461-472.
7. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Molecular microbiology **22:**703-714.

8. **Baltes, N., F. F. Buettner, and G. F. Gerlach.** 2007. Selective capture of transcribed sequences (SCOTS) of *Actinobacillus pleuropneumoniae* in the chronic stage of disease reveals an HlyX-regulated autotransporter protein. Veterinary microbiology **123:**110-121.

9. **Baltes, N., and G. F. Gerlach.** 2004. Identification of genes transcribed by *Actinobacillus pleuropneumoniae* in necrotic porcine lung tissue by using selective capture of transcribed sequences. Infection and immunity **72:**6711-6716.

10. **Bang, I. S., B. H. Kim, J. W. Foster, and Y. K. Park.** 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. Journal of bacteriology **182:**2245-2252.

11. **Bard, L.** 1918. Précis des examens de laboratoire, Masson & cie éditeurs, Paris.

12. **Basnyat, B.** 2007. The treatment of enteric fever. Journal of the Royal Society of Medicine **100:**161-162.

13. **Baumler, A. J., R. M. Tsolis, and F. Heffron.** 1996. The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. Proceedings of the National Academy of Sciences of the United States of America **93:**279-283.

14. **Baumler, A. J., R. M. Tsolis, A. W. van der Velden, I. Stojiljkovic, S. Anic, and F. Heffron.** 1996. Identification of a new iron regulated locus of *Salmonella typhi*. Gene **183:**207-213.

15. **Beier, D., and R. Gross.** 2006. Regulation of bacterial virulence by two-component systems. Current opinion in microbiology **9:**143-152.

16. **Beuzon, C. R., G. Banks, J. Deiwick, M. Hensel, and D. W. Holden.** 1999. pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella typhimurium*. Molecular microbiology **33:**806-816.

17. **Beuzon, C. R., S. Meresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot, and D. W. Holden.** 2000. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. The EMBO journal **19:**3235-3249.

18. **Bhan, M. K., R. Bahl, and S. Bhatnagar.** 2005. Typhoid and paratyphoid fever. Lancet **366:**749-762.

19. **Bijlsma, J. J., and E. A. Groisman.** 2005. The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. Molecular microbiology **57:**85-96.

20. **Bister, B., D. Bischoff, G. J. Nicholson, M. Valdebenito, K. Schneider, G. Winkelmann, K. Hantke, and R. D. Sussmuth.** 2004. The structure of salmochelins: C-glucosylated enterobactins of *Salmonella enterica*. Biometals **17:**471-481.

21. **Bjur, E., S. Eriksson-Ygberg, and M. Rhen.** 2006. The O-antigen affects replication of *Salmonella enterica* serovar Typhimurium in murine macrophage-like J774-A.1 cells through modulation of host cell nitric oxide production. Microbes and infection / Institut Pasteur **8:**1826-1838.

22. **Black, M. T., and J. Hodgson.** 2005. Novel target sites in bacteria for overcoming antibiotic resistance. Advanced drug delivery reviews **57:**1528-1538.

23. **Blanc-Potard, A. B., and E. A. Groisman.** 1997. The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. The EMBO journal **16:**5376-5385.

24. **Blanc-Potard, A. B., F. Solomon, J. Kayser, and E. A. Groisman.** 1999. The SPI-3 pathogenicity island of *Salmonella enterica.* Journal of bacteriology **181:**998-1004.

25. **Brogden, K. A.** 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nature reviews **3:**238-250.

26. **Brumell, J. H., C. M. Rosenberger, G. T. Gotto, S. L. Marcus, and B. B. Finlay.** 2001. SifA permits survival and replication of *Salmonella typhimurium* in murine macrophages. Cellular microbiology **3:**75-84.

27. **Buchmeier, N., S. Bossie, C. Y. Chen, F. C. Fang, D. G. Guiney, and S. J. Libby.** 1997. SlyA, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. Infection and immunity **65:**3725-3730.

28. **Bueno, S. M., C. A. Santiviago, A. A. Murillo, J. A. Fuentes, A. N. Trombert, P. I. Rodas, P. Youderian, and G. C. Mora.** 2004. Precise excision of the large pathogenicity island, SPI7, in *Salmonella enterica* serovar Typhi. Journal of bacteriology **186:**3202-3213.

29. **Castelli, M. E., E. Garcia Vescovi, and F. C. Soncini.** 2000. The phosphatase activity is the target for Mg2+ regulation of the sensor protein PhoQ in *Salmonella.* The Journal of biological chemistry **275:**22948-22954.

30. **Chan, K., C. C. Kim, and S. Falkow.** 2005. Microarray-based detection of *Salmonella enterica* serovar Typhimurium transposon mutants that cannot survive in macrophages and mice. Infection and immunity **73:**5438-5449.

31. **Chen, L. M., K. Kaniga, and J. E. Galan.** 1996. *Salmonella spp.* are cytotoxic for cultured macrophages. Molecular microbiology **21:**1101-1115.

32. **Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow.** 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Molecular microbiology **30:**175-188.

33. **Clark, D. P. a. C., J. E.** 1996. Two-Carbon Compounds and Fatty Acids as Carbon Sources. *In* F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology, 2nd Edition ed. ASM Press, Washington, D.C.

34. **Coburn, B., Y. Li, D. Owen, B. A. Vallance, and B. B. Finlay.** 2005. *Salmonella enterica* serovar Typhimurium pathogenicity island 2 is necessary for complete virulence in a mouse model of infectious enterocolitis. Infection and immunity **73:**3219-3227.

35. **Collins, H. L.** 2003. The role of iron in infections with intracellular bacteria. Immunology Letters **85:**193-195.

36. **Conner, C. P., D. M. Heithoff, S. M. Julio, R. L. Sinsheimer, and M. J. Mahan.** 1998. Differential patterns of acquired virulence genes distinguish *Salmonella* strains. Proceedings of the National Academy of Sciences of the United States of America **95:**4641-4645.

37. **Connor, B. A., and E. Schwartz.** 2005. Typhoid and paratyphoid fever in travellers. The Lancet infectious diseases **5:**623-628.

38. **Conway, T., and G. K. Schoolnik.** 2003. Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. Molecular microbiology **47:**879-889.

39. **Coombes, B. K., N. F. Brown, Y. Valdez, J. H. Brumell, and B. B. Finlay.** 2004. Expression and secretion of *Salmonella* pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL. The Journal of biological chemistry **279:**49804-49815.

40. **Coombes, B. K., M. J. Lowden, J. L. Bishop, M. E. Wickham, N. F. Brown, N. Duong, S. Osborne, O. Gal-Mor, and B. B. Finlay.** 2007. SseL is a *Salmonella*-specific translocated effector integrated into the SsrB-controlled salmonella pathogenicity island 2 type III secretion system. Infection and immunity **75:**574-580.

41. **Cotter, P. A., and J. F. Miller.** 1998. *In vivo* and *ex vivo* regulation of bacterial virulence gene expression. Current opinion in microbiology **1:**17-26.

42. **Crogan, N. L., and B. C. Evans.** 2007. *Clostridium difficile*: an emerging epidemic in nursing homes. Geriatric nursing **28:**161-164.

43. **Crump, J. A., S. P. Luby, and E. D. Mintz.** 2004. The global burden of typhoid fever. Bulletin of the World Health Organization **82:**346-353.

44. **Daigle, F., J. E. Graham, and R. Curtiss, 3rd.** 2001. Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). Molecular microbiology **41:**1211-1222.

45. **Daniels, J. J., I. B. Autenrieth, A. Ludwig, and W. Goebel.** 1996. The gene *slyA* of *Salmonella typhimurium* is required for destruction of M cells and intracellular survival but not for invasion or colonization of the murine small intestine. Infection and immunity **64:**5075-5084.

46. **Darwin, K. H., and V. L. Miller.** 2000. The putative invasion protein chaperone SicA acts together with InvF to activate the expression of *Salmonella typhimurium* virulence genes. Molecular microbiology **35:**949-960.

47. **Darwin, K. H., and V. L. Miller.** 2001. Type III secretion chaperone-dependent regulation: activation of virulence genes by SicA and InvF in *Salmonella typhimurium*. The EMBO journal **20:**1850-1862.

48. **Dathe, M., J. Meyer, M. Beyermann, B. Maul, C. Hoischen, and M. Bienert.** 2002. General aspects of peptide selectivity towards lipid bilayers and cell membranes studied by variation of the structural parameters of amphipathic helical model peptides. Biochimica et biophysica acta **1558:**171-186.

49. **Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel.** 1999. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. Molecular microbiology **31:**1759-1773.

50. **Deiwick, J., S. P. Salcedo, E. Boucrot, S. M. Gilliland, T. Henry, N. Petermann, S. R. Waterman, J. P. Gorvel, D. W. Holden, and S. Meresse.** 2006. The translocated *Salmonella* effector proteins SseF and SseG interact and are required to establish an intracellular replication niche. Infection and immunity **74:**6965-6972.

51. **DiPetrillo, M. D., T. Tibbetts, H. Kleanthous, K. P. Killeen, and E. L. Hohmann.** 1999. Safety and immunogenicity of *phoP*/*phoQ*-deleted *Salmonella*

typhi expressing *Helicobacter pylori* urease in adult volunteers. Vaccine **18:**449-459.

52. **Doerrler, W. T.** 2006. Lipid trafficking to the outer membrane of Gram-negative bacteria. Molecular microbiology **60:**542-552.

53. **Dorsey, C. W., M. C. Laarakker, A. D. Humphries, E. H. Weening, and A. J. Baumler.** 2005. *Salmonella enterica* serotype Typhimurium MisL is an intestinal colonization factor that binds fibronectin. Molecular microbiology **57:**196-211.

54. **Dozois, C. M., F. Daigle, and R. Curtiss, 3rd.** 2003. Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic *Escherichia coli* strain. Proceedings of the National Academy of Sciences of the United States of America **100:**247-252.

55. **Edwards, R. A., G. J. Olsen, and S. R. Maloy.** 2002. Comparative genomics of closely related *Salmonellae*. Trends in microbiology **10:**94-99.

56. **Edwards, R. A., D. M. Schifferli, and S. R. Maloy.** 2000. A role for *Salmonella* fimbriae in intraperitoneal infections. Proceedings of the National Academy of Sciences of the United States of America **97:**1258-1262.

57. **Ellermeier, C. D., J. R. Ellermeier, and J. M. Slauch.** 2005. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator *hilA* in *Salmonella enterica* serovar Typhimurium. Molecular microbiology **57:**691-705.

58. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Molecular microbiology **47:**103-118.

59. **Ernst, R. K., T. Guina, and S. I. Miller.** 2001. *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. Microbes and infection / Institut Pasteur **3:**1327-1334.

60. **Everest, P., J. Wain, M. Roberts, G. Rook, and G. Dougan.** 2001. The molecular mechanisms of severe typhoid fever. Trends in microbiology **9:**316-320.

61. **Fang, F. C., S. J. Libby, M. E. Castor, and A. M. Fung.** 2005. Isocitrate lyase (AceA) is required for *Salmonella* persistence but not for acute lethal infection in mice. Infection and immunity **73:**2547-2549.

62. **Faucher, S. P., S. Porwollik, C. M. Dozois, M. McClelland, and F. Daigle.** 2006. Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. Proceedings of the National Academy of Sciences of the United States of America **103:**1906-1911.

63. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proceedings of the National Academy of Sciences of the United States of America **83:**5189-5193.

64. **Fink, S. L., and B. T. Cookson.** 2006. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. Cellular microbiology **8:**1812-1825.

65. **Folkesson, A., S. Lofdahl, and S. Normark.** 2002. The *Salmonella enterica* subspecies I specific centisome 7 genomic island encodes novel protein families

present in bacteria living in close contact with eukaryotic cells. Research in Microbiology **153:**537-545.

66. **Fuller, T. E., R. J. Shea, B. J. Thacker, and M. H. Mulks.** 1999. Identification of in vivo induced genes in *Actinobacillus pleuropneumoniae*. Microbial pathogenesis **27:**311-327.

67. **Galan, J. E.** 1996. Molecular and cellular bases of *Salmonella* entry into host cells. Current topics in microbiology and immunology **209:**43-60.

68. **Galan, J. E., and R. Curtiss III.** 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infection and immunity **58:**1879-1885.

69. **Galan, J. E., and R. I. Curtiss.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proceedings of the National Academy of Sciences of the United States of America **86:**6383-6387.

70. **Galan, J. E., and H. Wolf-Watz.** 2006. Protein delivery into eukaryotic cells by type III secretion machines. Nature **444:**567-573.

71. **Ganz, T.** 2003. Defensins: antimicrobial peptides of innate immunity. Nature Reviews Immunology **3:**710-720.

72. **Ganz, T., M. E. Selsted, D. Szklarek, S. S. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer.** 1985. Defensins. Natural peptide antibiotics of human neutrophils. The Journal of clinical investigation **76:**1427-1435.

73. **Garcia-del Portillo, F., M. A. Stein, and B. B. Finlay.** 1997. Release of lipopolysaccharide from intracellular compartments containing *Salmonella*

typhimurium to vesicles of the host epithelial cell. Infection and immunity **65:**24-34.

74. **Garmendia, J., C. R. Beuzon, J. Ruiz-Albert, and D. W. Holden.** 2003. The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the *Salmonella typhimurium* SPI-2 type III secretion system. Microbiology **149:**2385-2396.

75. **Garmory, H. S., K. A. Brown, and R. W. Titball.** 2002. *Salmonella* vaccines for use in humans: present and future perspectives. FEMS microbiology reviews **26:**339-353.

76. **Gerlach, R. G., D. Jackel, B. Stecher, C. Wagner, A. Lupas, W. D. Hardt, and M. Hensel.** 2007. *Salmonella* Pathogenicity Island 4 encodes a giant non-fimbrial adhesin and the cognate type 1 secretion system. Cellular microbiology **9:**1834-1850.

77. **Ghosh, P.** 2004. Process of protein transport by the type III secretion system. Microbiololy and Molecular Biology Reviews **68:**771-795.

78. **Gibbons, H. S., S. R. Kalb, R. J. Cotter, and C. R. Raetz.** 2005. Role of Mg2+ and pH in the modification of *Salmonella* lipid A after endocytosis by macrophage tumour cells. Molecular microbiology **55:**425-440.

79. **Gophna, U., E. Z. Ron, and D. Graur.** 2003. Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. Gene **312:**151-163.

80. **Graham, J. E., and J. E. Clark-Curtiss.** 1999. Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS).

Proceedings of the National Academy of Sciences of the United States of America **96:**11554-11559.

81. **Graham, J. E., R. M. Peek, Jr., U. Krishna, and T. L. Cover.** 2002. Global analysis of *Helicobacter pylori* gene expression in human gastric mucosa. Gastroenterology **123:**1637-1648.

82. **Groisman, E. A.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. Journal of bacteriology **183:**1835-1842.

83. **Groisman, E. A., and H. Ochman.** 1996. Pathogenicity islands: bacterial evolution in quantum leaps. Cell **87:**791-794.

84. **Guina, T., E. C. Yi, H. Wang, M. Hackett, and S. I. Miller.** 2000. A PhoP-regulated outer membrane protease of *Salmonella enterica* serovar Typhimurium promotes resistance to alpha-helical antimicrobial peptides. Journal of bacteriology **182:**4077-4086.

85. **Gulig, P. A., and T. J. Doyle.** 1993. The *Salmonella typhimurium* virulence plasmid increases the growth rate of *Salmonellae* in mice. Infection and immunity **61:**504-511.

86. **Gunn, J. S., K. B. Lim, J. Krueger, K. Kim, L. Guo, M. Hackett, and S. I. Miller.** 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Molecular microbiology **27:**1171-1182.

87. **Gunzel, D., L. M. Kucharski, D. G. Kehres, M. F. Romero, and M. E. Maguire.** 2006. The MgtC virulence factor of *Salmonella enterica* serovar Typhimurium activates Na(+),K(+)-ATPase. Journal of bacteriology **188:**5586-5594.

88. **Guo, L., K. B. Lim, J. S. Gunn, B. Bainbridge, R. P. Darveau, M. Hackett, and S. I. Miller.** 1997. Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. Science **276:**250-253.

89. **Guo, L., K. B. Lim, C. M. Poduje, M. Daniel, J. S. Gunn, M. Hackett, and S. I. Miller.** 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell **95:**189-198.

90. **Guzman, C. A., S. Borsutzky, M. Griot-Wenk, I. C. Metcalfe, J. Pearman, A. Collioud, D. Favre, and G. Dietrich.** 2006. Vaccines against typhoid fever. Vaccine **24:**3804-3811.

91. **Haghjoo, E., and J. E. Galan.** 2004. *Salmonella typhi* encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. Proceedings of the National Academy of Sciences of the United States of America **101:**4614-4619.

92. **Hansen-Wester, I., D. Chakravortty, and M. Hensel.** 2004. Functional transfer of *Salmonella* pathogenicity island 2 to *Salmonella bongori* and *Escherichia coli*. Infection and immunity **72:**2879-2888.

93. **Hantke, K., G. Nicholson, W. Rabsch, and G. Winkelmann.** 2003. Salmochelins, siderophores of *Salmonella enterica* and uropathogenic *Escherichia coli* strains, are recognized by the outer membrane receptor IroN. Proceedings of the National Academy of Sciences of the United States of America **100:**3677-3682.

94. **Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galan.** 1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell **93:**815-826.

95. **Hashimoto, Y., N. Li, H. Yokoyama, and T. Ezaki.** 1993. Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. Journal of bacteriology **175:**4456-4465.

96. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by in vivo gene expression. Proceedings of the National Academy of Sciences of the United States of America **94:**934-939.

97. **Hensel, M.** 2004. Evolution of pathogenicity islands of *Salmonella enterica*. International Journal of Medical Microbiology **294:**95-102.

98. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400-403.

99. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden.** 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Molecular microbiology **30:**163-174.

100. **Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky.** 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proceedings of the National Academy of Sciences of the United States of America **96:**2396-2401.

101. **Hinton, J. C., I. Hautefort, S. Eriksson, A. Thompson, and M. Rhen.** 2004. Benefits and pitfalls of using microarrays to monitor bacterial gene expression during infection. Current opinion in microbiology **7:**277-282.

102. **Hirose, K., T. Ezaki, M. Miyake, T. Li, A. Q. Khan, Y. Kawamura, H. Yokoyama, and T. Takami.** 1997. Survival of Vi-capsulated and Vi-deleted *Salmonella typhi* strains in cultured macrophage expressing different levels of CD14 antigen. FEMS microbiology letters **147:**259-265.

103. **Hoare, A., M. Bittner, J. Carter, S. Alvarez, M. Zaldivar, D. Bravo, M. A. Valvano, and I. Contreras.** 2006. The outer core lipopolysaccharide of *Salmonella enterica* serovar Typhi is required for bacterial entry into epithelial cells. Infection and immunity **74:**1555-1564.

104. **Hohmann, E. L., C. A. Oletta, K. P. Killeen, and S. I. Miller.** 1996. *phoP/phoQ*-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. The Journal of infectious diseases **173:**1408-1414.

105. **Holden, D. W.** 2002. Trafficking of the *Salmonella* vacuole in macrophages. Traffic **3:**161-169.

106. **Hook, E. W.** 1985. *Salmonella* species (including typhoid fever), p.1256-1269. *In* R. G. D. a. J. E. B. e. G.L. Mandell (ed.), *In*: Principles and practices in infectious diseases. Wiley and Sons, NY.

107. **Hornick, R. B., S. E. Greisman, T. E. Woodward, H. L. DuPont, A. T. Dawkins, and M. J. Snyder.** 1970. Typhoid fever: pathogenesis and immunologic control. The New England journal of medicine **283:**686-691.

108. **Hou, J. Y., J. E. Graham, and J. E. Clark-Curtiss.** 2002. *Mycobacterium avium* genes expressed during growth in human macrophages detected by Selective Capture of Transcribed Sequences (SCOTS). Infection and immunity **70:**3714-3726.

109. **Hsing, W., and T. J. Silhavy.** 1997. Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli*. Journal of bacteriology **179:**3729-3735.

110. **Hudson, D. L., A. N. Layton, T. R. Field, A. J. Bowen, H. Wolf-Watz, M. Elofsson, M. P. Stevens, and E. E. Galyov.** 2007. Inhibition of type III secretion in *Salmonella enterica* serovar Typhimurium by small-molecule inhibitors. Antimicrobial agents and chemotherapy **51:**2631-2635.

111. **Humphries, A. D., M. Raffatellu, S. Winter, E. H. Weening, R. A. Kingsley, R. Droleskey, S. Zhang, J. Figueiredo, S. Khare, J. Nunes, L. G. Adams, R. M. Tsolis, and A. J. Baumler.** 2003. The use of flow cytometry to detect expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. Molecular microbiology **48:**1357-1376.

112. **Janakiraman, A., and J. M. Slauch.** 2000. The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella typhimurium*. Molecular microbiology **35:**1146-1155.

113. **Kammler, M., C. Schon, and K. Hantke.** 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. Journal of bacteriology **175:**6212-6219.

114. **Kato, A., and E. A. Groisman.** 2004. Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. Genes & development **18:**2302-2313.

115. **Kaufmann, S. H., B. Raupach, and B. B. Finlay.** 2001. Introduction: microbiology and immunology: lessons learned from *Salmonella*. Microbes and infection / Institut Pasteur **3:**1177-1181.

116. **Kehres, D. G., A. Janakiraman, J. M. Slauch, and M. E. Maguire.** 2002. SitABCD is the alkaline Mn(2+) transporter of *Salmonella enterica* serovar Typhimurium. Journal of bacteriology **184:**3159-3166.

117. **Khan, M. A., and R. E. Isaacson.** 2002. Identification of *Escherichia coli* genes that are specifically expressed in a murine model of septicemic infection. Infection and immunity **70:**3404-3412.

118. **Klemm, P., R. M. Vejborg, and O. Sherlock.** 2006. Self-associating autotransporters, SAATs: functional and structural similarities. International Journal of Medical Microbiology **296:**187-195.

119. **Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa.** 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science **280:**602-605.

120. **Kuehn, M. J., and N. C. Kesty.** 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes & development **19:**2645-2655.

121. **Lai, Y. C., H. L. Peng, and H. Y. Chang.** 2001. Identification of genes induced in vivo during *Klebsiella pneumoniae* CG43 infection. Infection and immunity **69:**7140-7145.

122. **Lalmanach, A. C., and F. Lantier.** 1999. Host cytokine response and resistance to *Salmonella infection*. Microbes and infection / Institut Pasteur **1:**719-726.

123. **Lamichhane, G., and W. Bishai.** 2007. Defining the 'survivasome' of *Mycobacterium tuberculosis*. Nature medicine **13:**280-282.

124. **Lawley, T. D., K. Chan, L. J. Thompson, C. C. Kim, G. R. Govoni, and D. M. Monack.** 2006. Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. PLoS pathogens **2:**e11.

125. **Ledeboer, N. A., J. G. Frye, M. McClelland, and B. D. Jones.** 2006. *Salmonella enterica* serovar Typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. Infection and immunity **74:**3156-3169.

126. **Lee, A. K., C. S. Detweiler, and S. Falkow.** 2000. OmpR regulates the two-component system SsrA-ssrB in *Salmonella* pathogenicity island 2. Journal of bacteriology **182:**771-781.

127. **Lee, F. K., C. Morris, and J. Hackett.** 2006. The *Salmonella enterica* serovar Typhi Vi capsule and self-association pili share controls on expression. FEMS microbiology letters **261:**41-46.

128. **Lee, S. H., and J. E. Galan.** 2004. *Salmonella* type III secretion-associated chaperones confer secretion-pathway specificity. Molecular microbiology **51:**483-495.

129. **Lehrer, R. I., A. Barton, K. A. Daher, S. S. Harwig, T. Ganz, and M. E. Selsted.** 1989. Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. The Journal of clinical investigation **84:**553-561.

130. **Lejona, S., A. Aguirre, M. L. Cabeza, E. Garcia Vescovi, and F. C. Soncini.** 2003. Molecular characterization of the Mg2+-responsive PhoP-PhoQ regulon in *Salmonella enterica*. Journal of bacteriology **185:**6287-6294.

131. **Li, Z., A. J. Clarke, and T. J. Beveridge.** 1998. Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. Journal of bacteriology **180:**5478-5483.

132. **Libby, S. J., W. Goebel, A. Ludwig, N. Buchmeier, F. Bowe, F. C. Fang, D. G. Guiney, J. G. Songer, and F. Heffron.** 1994. A cytolysin encoded by

Salmonella is required for survival within macrophages. Proceedings of the National Academy of Sciences of the United States of America **91:**489-493.

133. **Linehan, S. A., A. Rytkonen, X. J. Yu, M. Liu, and D. W. Holden.** 2005. SlyA Regulates Function of *Salmonella* Pathogenicity Island 2 (SPI-2) and Expression of SPI-2-Associated Genes. Infection and immunity **73:**4354-4362.

134. **Lostroh, C. P., and C. A. Lee.** 2001. The *Salmonella* pathogenicity island-1 type III secretion system. Microbes and infection / Institut Pasteur **3:**1281-1291.

135. **Lott, J. W.** 2006. State of the science: neonatal bacterial infection in the early 21st century. The Journal of perinatal & neonatal nursing **20:**62-70.

136. **Lucas, R. L., and C. A. Lee.** 2001. Roles of hilC and hilD in regulation of hilA expression in *Salmonella enterica* serovar Typhimurium. Journal of bacteriology **183:**2733-2745.

137. **Ludwig, A., S. Bauer, R. Benz, B. Bergmann, and W. Goebel.** 1999. Analysis of the SlyA-controlled expression, subcellular localization and pore-forming activity of a 34 kDa haemolysin (ClyA) from *Escherichia coli* K-12. Molecular microbiology **31:**557-567.

138. **Lundberg, U., U. Vinatzer, D. Berdnik, A. von Gabain, and M. Baccarini.** 1999. Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. Journal of bacteriology **181:**3433-3437.

139. **Ly, K. T., and J. E. Casanova.** 2007. Mechanisms of *Salmonella* entry into host cells. Cellular microbiology **9:**2103-2111.

140. **Lyczak, J. B., and G. B. Pier.** 2002. *Salmonella enterica* serovar Typhi modulates cell surface expression of its receptor, the cystic fibrosis

transmembrane conductance regulator, on the intestinal epithelium. Infection and immunity **70:**6416-6423.

141. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bactertial virulence genes that are specifically induced in host tissues. Science **259:**686-688.

142. **Mahan, M. J., J. W. Tobias, J. M. Slauch, P. C. Hanna, R. J. Collier, and J. J. Mekalanos.** 1995. Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. Proceedings of the National Academy of Sciences of the United States of America **92:**669-673.

143. **Martin-Orozco, N., N. Touret, M. L. Zaharik, E. Park, R. Kopelman, S. Miller, B. B. Finlay, P. Gros, and S. Grinstein.** 2006. Visualization of vacuolar acidification-induced transcription of genes of pathogens inside macrophages. Molecular biology of the cell **17:**498-510.

144. **Mashburn-Warren, L. M., and M. Whiteley.** 2006. Special delivery: vesicle trafficking in prokaryotes. Molecular microbiology **61:**839-846.

145. **McClelland, M., K. E. Sanderson, S. W. Clifton, P. Latreille, S. Porwollik, A. Sabo, R. Meyer, T. Bieri, P. Ozersky, M. McLellan, C. R. Harkins, C. Wang, C. Nguyen, A. Berghoff, G. Elliott, S. Kohlberg, C. Strong, F. Du, J. Carter, C. Kremizki, D. Layman, S. Leonard, H. Sun, L. Fulton, W. Nash, T. Miner, P. Minx, K. Delehaunty, C. Fronick, V. Magrini, M. Nhan, W. Warren, L. Florea, J. Spieth, and R. K. Wilson.** 2004. Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. Nature Genetics **36:**1268-1274.

146. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852-856.

147. **McKinney, J. D., K. Honer zu Bentrup, E. J. Munoz-Elias, A. Miczak, B. Chen, W. T. Chan, D. Swenson, J. C. Sacchettini, W. R. Jacobs, Jr., and D. G. Russell.** 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature **406:**735-738.

148. **Merrell, D. S., S. M. Butler, F. Qadri, N. A. Dolganov, A. Alam, M. B. Cohen, S. B. Calderwood, G. K. Schoolnik, and A. Camilli.** 2002. Host-induced epidemic spread of the cholera bacterium. Nature **417:**642-645.

149. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proceedings of the National Academy of Sciences of the United States of America **86:**5054-5058.

150. **Mills, D. M., V. Bajaj, and C. A. Lee.** 1995. A 40 kilobase chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. Molecular Microbiology **15:**749-759.

151. **Mirold, S., W. Rabsch, M. Rohde, S. Stender, H. Tschape, H. Russmann, E. Igwe, and W. D. Hardt.** 1999. Isolation of a temperate bacteriophage encoding

the type III effector protein SopE from an epidemic *Salmonella typhimurium* strain. Proceedings of the National Academy of Sciences of the United States of America **96:**9845-9850.

152. **Monack, D. M., D. M. Bouley, and S. Falkow.** 2004. *Salmonella typhimurium* persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization. The Journal of experimental medicine **199:**231-241.

153. **Monack, D. M., A. Mueller, and S. Falkow.** 2004. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nature reviews **2:**747-765.

154. **Montagne, M., A. Martel, and H. Le Moual.** 2001. Characterization of the catalytic activities of the PhoQ histidine protein kinase of *Salmonella enterica* serovar Typhimurium. Journal of bacteriology **183:**1787-1791.

155. **Morrow, B. J., J. E. Graham, and R. Curtiss, 3rd.** 1999. Genomic subtractive hybridization and selective capture of transcribed sequences identify a novel *Salmonella typhimurium* fimbrial operon and putative transcriptional regulator that are absent from the *Salmonella typhi* genome. Infection and immunity **67:**5106-5116.

156. **Mullen, L. M., S. P. Nair, J. M. Ward, A. N. Rycroft, and B. Henderson.** 2006. Phage display in the study of infectious diseases. Trends in microbiology **14:**141-147.

157. **Murray, G. L., S. R. Attridge, and R. Morona.** 2006. Altering the length of the lipopolysaccharide O antigen has an impact on the interaction of *Salmonella*

enterica serovar Typhimurium with macrophages and complement. Journal of bacteriology **188:**2735-2739.

158. **Murray, G. L., S. R. Attridge, and R. Morona.** 2003. Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. Molecular microbiology **47:**1395-1406.

159. **Nair, S., S. Alokam, S. Kothapalli, S. Porwollik, E. Proctor, C. Choy, M. McClelland, S. L. Liu, and K. E. Sanderson.** 2004. *Salmonella enterica* serovar Typhi strains from which SPI7, a 134-kilobase island with genes for Vi exopolysaccharide and other functions, has been deleted. Journal of bacteriology **186:**3214-3223.

160. **Navarre, W. W., T. A. Halsey, D. Walthers, J. Frye, M. McClelland, J. L. Potter, L. J. Kenney, J. S. Gunn, F. C. Fang, and S. J. Libby.** 2005. Co-regulation of *Salmonella enterica* genes required for virulence and resistance to antimicrobial peptides by SlyA and PhoP/PhoQ. Molecular microbiology **56:**492-508.

161. **Negrea, A., E. Bjur, S. E. Ygberg, M. Elofsson, H. Wolf-Watz, and M. Rhen.** 2007. Salicylidene acylhydrazides that affect type III protein secretion in *Salmonella enterica* serovar Typhimurium. Antimicrobial agents and chemotherapy **51:**2867-2876.

162. **Ochman, H., and E. A. Groisman.** 1996. Distribution of pathogenicity islands in *Salmonella spp.* Infection and immunity **64:**5410-5412.

163. **Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman.** 1996. Identification of a pathogenicity island required for *Salmonella* survival in host

cells. Proceedings of the National Academy of Sciences of the United States of America **93:**7800-7804.

164. **Oscarsson, J., Y. Mizunoe, B. E. Uhlin, and D. J. Haydon.** 1996. Induction of haemolytic activity in *Escherichia coli* by the *slyA* gene product. Molecular microbiology **20:**191-199.

165. **Pan, Q., X. L. Zhang, H. Y. Wu, P. W. He, F. Wang, M. S. Zhang, J. M. Hu, B. Xia, and J. Wu.** 2005. Aptamers that preferentially bind type IVB pili and inhibit human monocytic-cell invasion by *Salmonella enterica* serovar Typhi. Antimicrobial agents and chemotherapy **49:**4052-4060.

166. **Papagrigorakis, M. J., C. Yapijakis, P. N. Synodinos, and E. Baziotopoulou-Valavani.** 2006. DNA examination of ancient dental pulp incriminates typhoid fever as a probable cause of the Plague of Athens. International Journal of Infectious Diseases **10:**206-214.

167. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848-852.

168. **Parry, C. M., T. T. Hien, G. Dougan, N. J. White, and J. J. Farrar.** 2002. Typhoid fever. The New England journal of medicine **347:**1770-1782.

169. **Perrin, A. J., X. Jiang, C. L. Birmingham, N. S. So, and J. H. Brumell.** 2004. Recognition of bacteria in the cytosol of Mammalian cells by the ubiquitin system. Current Biology **14:**806-811.

170. **Pickard, D., J. Li, M. Roberts, D. Maskell, D. Hone, M. Levine, G. Dougan, and S. Chatfield.** 1994. Characterization of defined *ompR* mutants of *Salmonella typhi*: *ompR* is involved in the regulation of Vi polysaccharide expression. Infection and immunity **62:**3984-3993.

171. **Pickard, D., J. Wain, S. Baker, A. Line, S. Chohan, M. Fookes, A. Barron, P. O. Gaora, J. A. Chabalgoity, N. Thanky, C. Scholes, N. Thomson, M. Quail, J. Parkhill, and G. Dougan.** 2003. Composition, acquisition, and distribution of the Vi exopolysaccharide-encoding *Salmonella enterica* pathogenicity island SPI-7. Journal of bacteriology **185:**5055-5065.

172. **Porwollik, S., E. F. Boyd, C. Choy, P. Cheng, L. Florea, E. Proctor, and M. McClelland.** 2004. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. Journal of bacteriology **186:**5883-5898.

173. **Porwollik, S., J. Frye, L. D. Florea, F. Blackmer, and M. McClelland.** 2003. A non-redundant microarray of genes for two related bacteria. Nucleic Acids Research **31:**1869-1876.

174. **Prouty, A. M., and J. S. Gunn.** 2000. *Salmonella enterica* serovar Typhimurium invasion is repressed in the presence of bile. Infection and immunity **68:**6763-6769.

175. **Raffatellu, M., D. Chessa, R. P. Wilson, R. Dusold, S. Rubino, and A. J. Baumler.** 2005. The Vi capsular antigen of *Salmonella enterica* serotype Typhi

reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. Infection and immunity **73:**3367-3374.

176. **Raffatellu, M., D. Chessa, R. P. Wilson, C. Tukel, M. Akcelik, and A. J. Baumler.** 2006. Capsule-mediated immune evasion: a new hypothesis explaining aspects of typhoid fever pathogenesis. Infection and immunity **74:**19-27.

177. **Raffatellu, M., R. L. Santos, D. Chessa, R. P. Wilson, S. Winter, C. A. Rossetti, S. D. Lawhon, H. Chu, T. Lau, C. L. Bevins, L. G. Adams, and A. J. Baumler.** 2007. The Capsule encoding *viaB* locus reduces IL-17 expression and mucosal innate responses in the bovine intestinal mucosa during infection with *Salmonella enterica* serotype Typhi. Infection and immunity, in press.

178. **Raffatellu, M., R. P. Wilson, D. Chessa, H. Andrews-Polymenis, Q. T. Tran, S. Lawhon, S. Khare, L. G. Adams, and A. J. Baumler.** 2005. SipA, SopA, SopB, SopD, and SopE2 contribute to *Salmonella enterica* serotype Typhimurium invasion of epithelial cells. Infection and immunity **73:**146-154.

179. **Randi, G., S. Franceschi, and C. La Vecchia.** 2006. Gallbladder cancer worldwide: geographical distribution and risk factors. International journal of cancer **118:**1591-1602.

180. **Ratledge, C., and L. G. Dover.** 2000. Iron metabolism in pathogenic bacteria. Annual review of microbiology **54:**881-941.

181. **Revel, A. T., A. M. Talaat, and M. V. Norgard.** 2002. DNA microarray analysis of differential gene expression in *Borrelia burgdorferi*, the Lyme disease spirochete. Proceedings of the National Academy of Sciences of the United States of America **99:**1562-1567.

182. **Richter-Dahlfors, A., A. M. Buchan, and B. B. Finlay.** 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. The Journal of experimental medicine **186:**569-580.

183. **Roland, K. L., L. E. Martin, C. R. Esther, and J. K. Spitznagel.** 1993. Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. Journal of bacteriology **175:**4154-4164.

184. **Rychlik, I., and P. A. Barrow.** 2005. *Salmonella* stress management and its relevance to behaviour during intestinal colonisation and infection. FEMS microbiology reviews **29:**1021-1040.

185. **Rytkonen, A., J. Poh, J. Garmendia, C. Boyle, A. Thompson, M. Liu, P. Freemont, J. C. Hinton, and D. W. Holden.** 2007. SseL, a *Salmonella* deubiquitinase required for macrophage killing and virulence. Proceedings of the National Academy of Sciences of the United States of America **104:**3502-3507.

186. **Salazar-Gonzalez, R. M., and S. J. McSorley.** 2005. *Salmonella* flagellin, a microbial target of the innate and adaptive immune system. Immunology Letters **101:**117-122.

187. **Salcedo, S. P., M. Noursadeghi, J. Cohen, and D. W. Holden.** 2001. Intracellular replication of *Salmonella typhimurium* strains in specific subsets of splenic macrophages in vivo. Cellular microbiology **3:**587-597.

188. **Santander, J., S. Y. Wanda, C. A. Nickerson, and R. Curtiss, 3rd.** 2007. Role of RpoS in fine-tuning the synthesis of Vi capsular polysaccharide in *Salmonella enterica* serotype Typhi. Infection and immunity **75:**1382-1392.

189. **Sarkar, N.** 1997. Polyadenylation of mRNA in prokaryotes., p. 173-197. *In* C. C. Richardson, Raetz, J.N.A., Raetz, C.R.H. and Thorner, J.W. (ed.), Annual reviews of biochemistry. Palo Alto, CA.

190. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin.** 2001. Comprehensive identification of conditionally essential genes in mycobacteria. Proceedings of the National Academy of Sciences of the United States of America **98:**12712-12717.

191. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Molecular microbiology **48:**77-84.

192. **Schmidt, H., and M. Hensel.** 2004. Pathogenicity islands in bacterial pathogenesis. Clinical microbiology reviews **17:**14-56.

193. **Schwan, W. R., X. Z. Huang, L. Hu, and D. J. Kopecko.** 2000. Differential bacterial survival, replication, and apoptosis-inducing ability of *Salmonella* serovars within human and murine macrophages. Infection and immunity **68:**1005-1013.

194. **Shotland, Y., H. Kramer, and E. A. Groisman.** 2003. The *Salmonella* SpiC protein targets the mammalian Hook3 protein function to alter cellular trafficking. Molecular microbiology **49:**1565-1576.

195. **Smith, J., R. Leke, A. Adams, and R. H. Tangermann.** 2004. Certification of polio eradication: process and lessons learned. Bulletin of the World Health Organization **82:**24-30.

196. **Snavely, M. D., C. G. Miller, and M. E. Maguire.** 1991. The *mgtB* Mg2+ transport locus of *Salmonella typhimurium* encodes a P-type ATPase. The Journal of biological chemistry **266:**815-823.

197. **Snyder, J. A., B. J. Haugen, E. L. Buckles, C. V. Lockatell, D. E. Johnson, M. S. Donnenberg, R. A. Welch, and H. L. Mobley.** 2004. Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. Infection and immunity **72:**6373-6381.

198. **Spory, A., A. Bosserhoff, C. von Rhein, W. Goebel, and A. Ludwig.** 2002. Differential regulation of multiple proteins of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium by the transcriptional regulator SlyA. Journal of bacteriology **184:**3549-3559.

199. **Starr, R., T. A. Willson, E. M. Viney, L. J. Murray, J. R. Rayner, B. J. Jenkins, T. J. Gonda, W. S. Alexander, D. Metcalf, N. A. Nicola, and D. J. Hilton.** 1997. A family of cytokine-inducible inhibitors of signalling. Nature **387:**917-921.

200. **Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold, and W. D. Hardt.** 2000. Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Molecular microbiology **36:**1206-1221.

201. **Szu, S. C., and S. Bystricky.** 2003. Physical, chemical, antigenic, and immunologic characterization of polygalacturonan, its derivatives, and Vi antigen from *Salmonella typhi*. Methods in enzymology **363:**552-567.

202. **Talaat, A. M., R. Lyons, S. T. Howard, and S. A. Johnston.** 2004. The temporal expression profile of *Mycobacterium tuberculosis* infection in mice.

Proceedings of the National Academy of Sciences of the United States of America **101:**4602-4607.

203. **Taylor, R. K., M. N. Hall, L. Enquist, and T. J. Silhavy.** 1981. Identification of OmpR: a positive regulatory protein controlling expression of the major outer membrane matrix porin proteins of *Escherichia coli* K-12. Journal of bacteriology **147:**255-258.

204. **Thomson, N., S. Baker, D. Pickard, M. Fookes, M. Anjum, N. Hamlin, J. Wain, D. House, Z. Bhutta, K. Chan, S. Falkow, J. Parkhill, M. Woodward, A. Ivens, and G. Dougan.** 2004. The role of prophage-like elements in the diversity of *Salmonella enterica* serovars. Journal of Molecular Biology **339:**279-300.

205. **Tierrez, A., and F. Garcia-del Portillo.** 2005. New concepts in *Salmonella* virulence: the importance of reducing the intracellular growth rate in the host. Cellular microbiology **7:**901-909.

206. **Tobar, J. A., L. J. Carreno, S. M. Bueno, P. A. Gonzalez, J. E. Mora, S. A. Quezada, and A. M. Kalergis.** 2006. Virulent *Salmonella enterica* serovar Typhimurium evades adaptive immunity by preventing dendritic cells from activating T cells. Infection and immunity **74:**6438-6448.

207. **Townsend, S. M., N. E. Kramer, R. Edwards, S. Baker, N. Hamlin, M. Simmonds, K. Stevens, S. Maloy, J. Parkhill, G. Dougan, and A. J. Baumler.** 2001. *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. Infection and immunity **69:**2894-2901.

208. **Tsui, I. S., C. M. Yip, J. Hackett, and C. Morris.** 2003. The type IVB pili of *Salmonella enterica* serovar Typhi bind to the cystic fibrosis transmembrane conductance regulator. Infection and immunity **71:**6049-6050.

209. **Tukel, C., M. Akcelik, M. F. de Jong, O. Simsek, R. M. Tsolis, and A. J. Baumler.** 2007. MarT activates expression of the MisL autotransporter protein of *Salmonella enterica* serotype Typhimurium. Journal of bacteriology **189:**3922-3926.

210. **Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman.** 1999. A *Salmonella* virulence protein that inhibits cellular trafficking. The EMBO journal **18:**3924-3933.

211. **Uchiya, K., and T. Nikai.** 2005. *Salmonella* pathogenicity island 2-dependent expression of suppressor of cytokine signaling 3 in macrophages. Infection and immunity **73:**5587-5594.

212. **van der Velden, A. W., S. W. Lindgren, M. J. Worley, and F. Heffron.** 2000. *Salmonella* pathogenicity island 1-independent induction of apoptosis in infected macrophages by *Salmonella enterica* serotype Typhimurium. Infection and immunity **68:**5702-5709.

213. **Vescovi, E. G., Y. M. Ayala, E. Di Cera, and E. A. Groisman.** 1997. Characterization of the bacterial sensor protein PhoQ. Evidence for distinct binding sites for Mg2+ and Ca2+. The Journal of biological chemistry **272:**1440-1443.

214. **Virlogeux, I., H. Waxin, C. Ecobichon, J. O. Lee, and M. Y. Popoff.** 1996. Characterization of the *rcsA* and *rcsB* genes from *Salmonella typhi*: *rcsB* through

tviA is involved in regulation of Vi antigen synthesis. Journal of bacteriology **178:**1691-1698.

215. **Virlogeux, I., H. Waxin, C. Ecobichon, and M. Y. Popoff.** 1995. Role of the viaB locus in synthesis, transport and expression of *Salmonella typhi* Vi antigen. Microbiology **141 (Pt 12):**3039-3047.

216. **Wain, J., V. B. Pham, V. Ha, N. M. Nguyen, S. D. To, A. L. Walsh, C. M. Parry, R. P. Hasserjian, V. A. HoHo, T. H. Tran, J. Farrar, N. J. White, and N. P. Day.** 2001. Quantitation of bacteria in bone marrow from patients with typhoid fever: relationship between counts and clinical features. Journal of clinical microbiology **39:**1571-1576.

217. **Wanachiwanawin, W.** 2000. Infections in E-beta thalassemia. Journal of Pediatric Hematology and Oncology **22:**581-587.

218. **Wattiau, P., and G. R. Cornelis.** 1993. SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in Ohe secretion of YopE. Molecular microbiology **8:**123-131.

219. **Weening, E. H., J. D. Barker, M. C. Laarakker, A. D. Humphries, R. M. Tsolis, and A. J. Baumler.** 2005. The *Salmonella enterica* serotype Typhimurium *lpf*, *bcf*, *stb*, *stc*, *std*, and *sth* fimbrial operons are required for intestinal persistence in mice. Infection and immunity **73:**3358-3366.

220. **Weissman, S. J., S. L. Moseley, D. E. Dykhuizen, and E. V. Sokurenko.** 2003. Enterobacterial adhesins and the case for studying SNPs in bacteria. Trends in microbiology **11:**115-117.

221. **Worley, M. J., G. S. Nieman, K. Geddes, and F. Heffron.** 2006. *Salmonella typhimurium* disseminates within its host by manipulating the motility of infected

cells. Proceedings of the National Academy of Sciences of the United States of America **103:**17915-17920.

222. **Wosten, M. M., L. F. Kox, S. Chamnongpol, F. C. Soncini, and E. A. Groisman.** 2000. A signal transduction system that responds to extracellular iron. Cell **103:**113-125.

223. **Young, V. B., S. Falkow, and G. K. Schoolnik.** 1992. The invasin protein of *Yersinia enterocolitica*: internalization of invasin-bearing bacteria by eukaryotic cells is associated with reorganization of the cytoskeleton. The Journal of cell biology **116:**197-207.

224. **Yu, X. J., M. Liu, and D. W. Holden.** 2004. SsaM and SpiC interact and regulate secretion of *Salmonella* pathogenicity island 2 type III secretion system effectors and translocators. Molecular microbiology **54:**604-619.

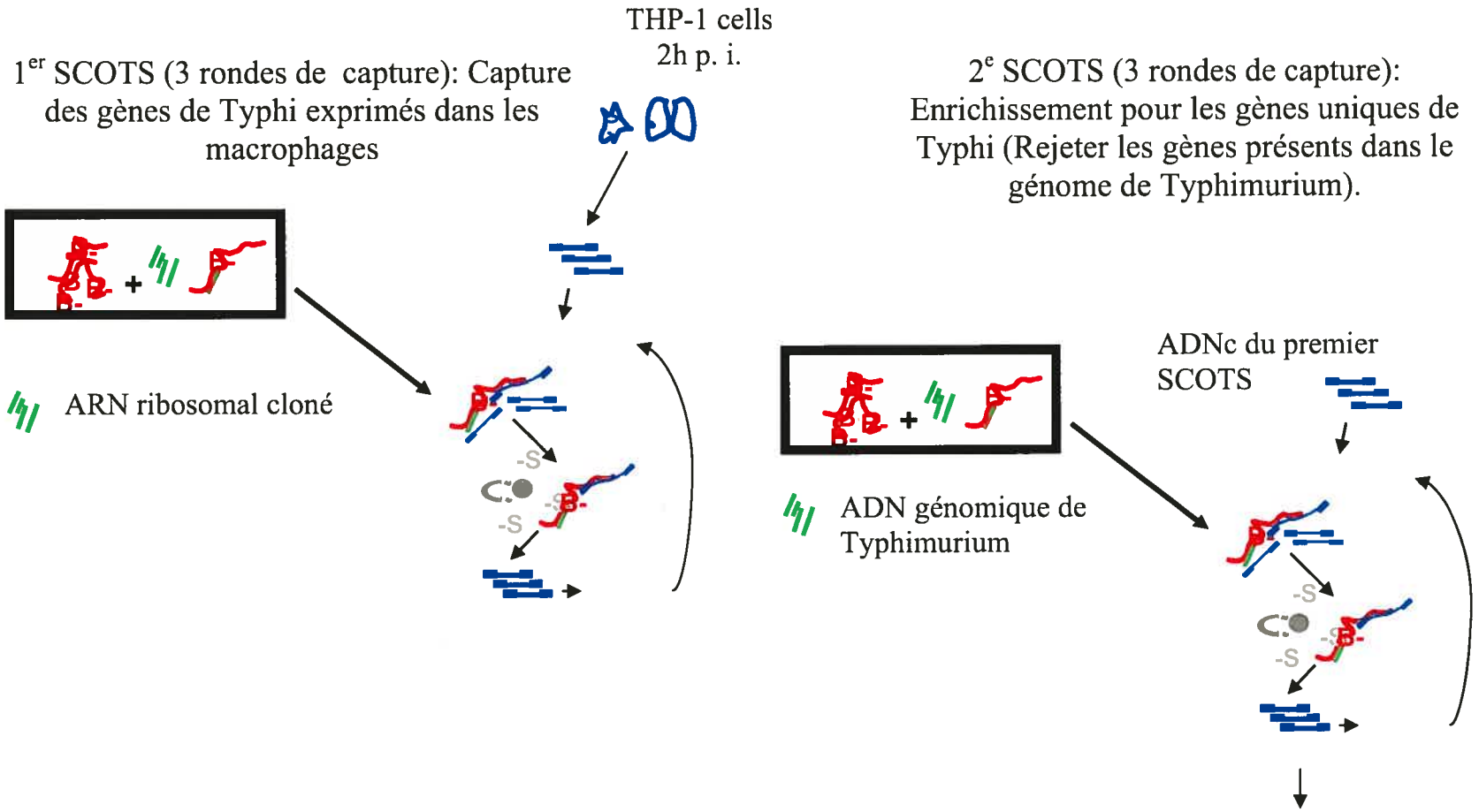
225. **Zanetti, M., R. Gennaro, and D. Romeo.** 1995. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS letters **374:**1-5.

226. **Zhang, S., R. L. Santos, R. M. Tsolis, S. Mirold, W. D. Hardt, L. G. Adams, and A. J. Baumler.** 2002. Phage mediated horizontal transfer of the *sopE1* gene increases enteropathogenicity of *Salmonella enterica* serotype Typhimurium for calves. FEMS microbiology letters **217:**243-247.

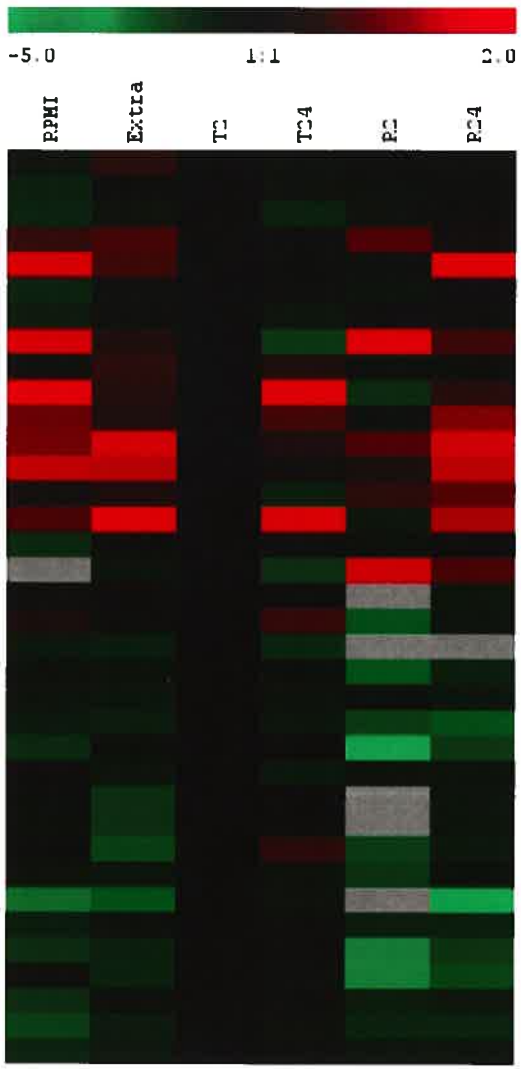
227. **Zhang, S., R. L. Santos, R. M. Tsolis, S. Stender, W. D. Hardt, A. J. Baumler, and L. G. Adams.** 2002. The *Salmonella* enterica serotype Typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves. Infection and immunity **70:**3843-3855.

228. **Zhang, X. L., I. S. Tsui, C. M. Yip, A. W. Fung, D. K. Wong, X. Dai, Y. Yang, J. Hackett, and C. Morris.** 2000. *Salmonella enterica* serovar Typhi uses type IVB pili to enter human intestinal epithelial cells. Infection and immunity **68:**3067-3073.

229. **Zhou, Z., A. A. Ribeiro, S. Lin, R. J. Cotter, S. I. Miller, and C. R. Raetz.** 2001. Lipid A modifications in polymyxin-resistant *Salmonella typhimurium*: PMRA-dependent 4-amino-4-deoxy-L-arabinose, and phosphoethanolamine incorporation. The Journal of Biological Chemistry **276:**43111-43121.



A1: Procédure expérimentale utilisée au chapitre 2



A2: Expression relatives des 36 gènes uniques de Typhi capturés par SCOTS

1) STY0115 putative IS transposase
2) STY0206 staB, chaperone protein EcpD precursor
2) STY0207 staA, putative fimbrial protein
3) STY1070 putative bacteriophage protein
4) STY1635 putative bacteriophage baseplate
4) STY1643 DNA-invertase
5) STY2005 conserved hypothetical protein
5) STY2020 putative bacteriophage protein
5) STY2022 putative exported protein
5) STY2026 putative bacteriophage protein
6) STY2732 putative regulatory protein (pseudo)
7) STY3068 hypothetical protein
8) STY3280 bacteriocin fragment (pseudo)
9) STY3663 hypothetical protein
10) STY3695 pin, DNA-invertase
11) STY3845 yiiF, putative CopG-family DNA-binding protein
12) STY3920 stgC, husher
13) STY3948 hypothetical protein
13) STY3950 hypothetical protein
14) STY4221 putative aminotransferase
15) STY4412 DeoR family regulatory protein
16) STY4526 hypothetical protein
16) STY4530 topB, topoisomerase B
16) STY4534 hypothetical protein
16) STY4536 ssb, single strand binding protein
16) STY4539 pilL, putative exported protein
16) STY4573 hypothetical protein
16) STY4574 hypothetical protein
16) STY4589 hypothetical protein
16) STY4643 apl, phage regulatory protein
16) STY4650 yjhP, conserved hypothetical protein
16) STY4654 vexB, Vi polysaccharide export
16) STY4656 tviE, Vi polysaccharide biosynthesis
16) STY4664 putative DNA helicase
16) STY4669a
17) STY4848 puatative transposase

A3: Protocoles relatifs aux biopuces.

Marquages de l'ADNc et de l'ADNg. L'ADNc obtenu par SCOTS a été marqué avec le fluorochrome Cy5. L'ADNg, utilisé comme référence a été marqué avec le fluorochrome Cy3. Dans les deux cas, 1,5 µg d'ADN a été marqué, selon le protocole de Patrick Brown (http://cmgm.stanford.edu/pbrown/protocols/4_genomic.html), en utilisant 10 U de la polymérase Klenow, 12µg d'hexamères aléatoires et 2 nmol de Cy3-dCTP ou Cy5-dCTP, pendant 16 h à 37 °C. L'ADN ainsi marqué a été purifié avec la trousse QIAquick PCR Purification (QIAgen) selon le protocole du fabricant.

Hybridation. Les biopuces sont préhybridées pendant 2 h à 42 °C dans une solution contenant 25% formamide, 5x SSC, 0.1% SDS et 1% ASB. Les biopuces sont ensuite rincées 2 fois dans une solution de 2x SSC et une fois dans de l'eau distillée. L'ADNc marqué est mélangé avec l'ADNg marqué et ajouté à une solution contenant 50 % formamide, 10x SSC et 0,2% SDS. L'ADN est ensuite dénaturé en chauffant à 99 °C pendant 5 minutes. Cette mixture est ensuite placée entre la biopuce et une lamelle de verre. La biopuce est incubée pendant 16 h à 42 °C dans une chambre à hybridation (Corning). Par la suite, la biopuce est lavée pendant 5 min dans une solution contenant 2x SSC et 0,1% SDS à 42 °C. Un autre lavage est effectué dans une solution de 1x SSC 0,1% SDS puis 4 lavages de 1 min dans 0,1x SSC. La biopuce est ensuite rincée à l'eau puis à l'éthanol. Elle est ensuite séchée par centrifugation à 400 *g*.

Acquisition des données et Normalisation. Les images de fluorescence ont été acquises à l'aide de l'appareil ScanArray Lite et du logiciel Scan Array Express 2.1

(Perkin Elmer). La quantification du signal a été réalisée à l'aide du logiciel QuantArray 3.0 (Perkin Elmer). Les données ont ensuite été normalisées en divisant le signal obtenu pour une sonde donnée par la somme du signal de toutes les sondes.

A4 : Autres contributions.

J'ai aussi contribué à ces deux autres articles qui sont présentés dans les pages suivantes.

1. **Abromaitis, S., S. Faucher, M. Beland, R. Curtiss, 3rd, and F. Daigle.** 2005. The presence of the tet gene from cloning vectors impairs Salmonella survival in macrophages. FEMS microbiology letters **242:**305-312.

Cet article présente une analyse de l'effet de certains plasmides sur la survie de *Salmonella* dans les macrophages. Nous avons démontré que le gène de résistance à la tétracycline (*tet*), codé sur certain vecteur, réduit la survie de *Salmonella* dans les macrophages.

2. **Faucher, S. P., S. Porwollik, C. M. Dozois, M. McClelland, and F. Daigle.** 2006. [Salmonella transcriptome during macrophage infection]. Medecine Science **22**:792-793.

Cet article, publié en français, présente les données du transcriptome de Typhi dans les biopuces.

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The presence of the *tet* gene from cloning vectors impairs *Salmonella* survival in macrophages

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Abstract

Cloning, mutagenesis and complementation of virulence factors are key steps to understand the mechanisms of bacterial pathogenesis and cloning vectors are routinely utilized for these processes. We have investigated the effect of the presence of commonly used cloning vectors on the survival of the intracellular bacterial pathogen *Salmonella* during macrophage infection. We demonstrate that the presence of the pSC101 derived tetracycline resistance gene on plasmids causes a lower survival rate of *Salmonella* in macrophages. The decrease in survival caused by the presence of the *tet* gene was not due to a higher susceptibility to gentamicin, a growth defect, or to increased sensitivity to acid. Higher susceptibility to hydrogen peroxide was observed in vitro for strain containing plasmid with the *tet* gene when the strains were grown at high densities but not when they were grown at low densities. Our findings demonstrate that the use of the *tet* gene for mutation or complementation can have deleterious effects and should thus be carefully considered.

Keywords: Plasmid effect; Tetracycline; *Salmonella*; Survival; Pathogenesis

1. Introduction

Application of newly developed molecular techniques to microbial systems has brought our understanding of bacterial pathogenesis to new heights. The growing number of complete bacterial genome sequences available provides unique insights into bacterial virulence, and can serve as a guide for using mutagenesis and in vivo techniques to identify key virulence genes. Virulence genes can be further characterized by other methods such as differential expression, either at the level of transcription (transcriptome) or at the level of protein expression (proteome). The information obtained from these approaches enables us to define an organism's virulome, which is the assembly of factors a pathogen requires for virulence. Characterization and understanding of a pathogen's virulome is key to develop new strategies for vaccination and novel antimicrobials.

As genomic information rapidly increases, the challenge remains to accurately understand how individual bacterial genes collaborate to cause infectious disease.

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Cloning vectors are widely utilized in the various steps of characterizing a gene (cloning, mutagenesis and complementation). Since plasmids have become frequently used genetic tools for cloning and expressing recombinant DNA, a growing number of reports showing the effects of plasmids on bacteria have been published [1–5]. It is well known that cloning vectors can lead to copy number artifacts by gene dosage effects. Moreover, the metabolic cost associated with the maintenance of plasmid functions can reduce bacterial fitness. A number of studies have shown that even in the absence of selection for plasmid-encoded functions, such as antibiotic resistance, plasmid carriage can reduce host fitness [6–10]. Thus plasmid maintenance, the vector itself without insert, can have pleiotropic effects on bacteria.

Cultured mammalian cells are commonly used to provide a controlled system for investigating mechanisms of bacterial adherence and invasion of host cells. *Salmonella enterica* is a pathogen that has been used extensively as a model organism for studying bacterial-host interactions [11]. *Salmonella* can colonize, invade and survive in its host while retaining plasmids. In this report, we have investigated the effect of commonly used cloning vectors on the survival of *Salmonella* during macrophage infection.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table 1. For macrophage infection, bacterial strains were routinely grown overnight in Luria–Bertani (LB) broth at 37 °C without shaking (standing) to an OD_{600} of 0.6 ($\approx 3 \times 10^8$ ml^{-1}), or when indicated, bacterial strains were grown to log phase with aeration, to an OD_{600} of 0.6. For bacterial growth measurement, overnight cultures were diluted 100-fold into fresh LB, complete cell culture medium RPMI (see below) or minimal M9 medium supplemented with 0.4% of either glucose or glycerol and amino acids (arginine at 22 $\mu g ml^{-1}$, cysteine at 22 $\mu g ml^{-1}$ and tryptophan at 20 $\mu g ml^{-1}$). At appropriate times, aliquots from the cultures were monitored for absorbance at 600 nm. Antibiotics when required were used at the following final concentrations: chloramphenicol (Cm) (30 $\mu g ml^{-1}$), kanamycin (Kan) (50 $\mu g ml^{-1}$), ampicilin (Ap) (50 $\mu g ml^{-1}$) and tetracycline (Tet) (10 $\mu g ml^{-1}$).

2.2. Cell culture and macrophage infection

The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 (Invitrogen) containing 10% fetal calf serum (FCS), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7) (Sigma), 2 mM L-glutamine, 1% MEM (modified Eagle's medium) non-essential amino acids (Sigma) and 1 mM of sodium pyruvate (Sigma). A stock culture of these cells was maintained as monocyte-like, non-adherent cells at 37 °C in an atmosphere containing 5% CO_2. Before infection, THP-1 cells were differentiated by addition of 10^{-7} M phorbol 12-myristate 13-acetate (PMA) for 24–48 h. Cells of the murine macrophage-like cell line RAW264.7 [12] were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FCS, 25 mM HEPES and 2 mM glutamine at 37 °C in a 5% CO_2 environment. For macrophage infection assays, cells were seeded at 5×10^5 cells per well in 24-well tissue culture dishes. Bacteria were added to the cell monolayer at a multiplicity of infection (m.o.i.) of 10:1, which does not affect cell viability (data not shown) and centrifuged for 5 min at 800*g* to synchronize phagocytosis. After incubation for 20 min at 37 °C (T_0), the infected cells were washed three times with prewarmed PBS and incubated with supplemented media as above containing 100 $\mu g ml^{-1}$ of gentamicin or polymyxin when indicated, to kill extracellular bacteria. The infected monolayers were either lysed from the tissue culture dishes by addition of 1 ml 0.1% sodium deoxycholate (DOC) in PBS per well or further incubated for 24 h with 12 $\mu g ml^{-1}$ of gentamicin. After lysis the number of surviving bacteria was determined as colony forming units (CFU) by plating on LB agar and, when necessary, on LB agar containing appropriate

Table 1
Bacterial strains and plasmids

Strain	Description	Origin of replication	Antibiotic resistance	Reference or source
χ3744	*S. typhi* ISP1820			D.M. Hone
Plasmids				
pACYC177	Cloning vector	p15A	Ap, Kan	[18]
pACYC184	Cloning vector	p15A	Cm, Tet	[18]
pBeloBAC11	Bacterial artificial chromosome vector	oriS	Cm	[42]
pBR322	Cloning vector	pMB1	Ap, Tet	[17]
pWSK29	Cloning vector	pSC101	Ap	[43]
pSC101	Cloning vector	pSC101	Tet	[19]
pSIF009	pACYC184 Δ*tet*	p15A	Cm	This work
pSIF010	pACYC177::*tet*	p15A	Kan, Tet	This work

antibiotics. All assays were conducted in duplicate and repeated independently at least three times. Results are expressed as the mean ± standard error of the replicate experiments. Percent phagocytosis was calculated by (CFU released by 0.1% DOC at T_0/CFU in the initial inoculum of bacteria added to each well) × 100. The survival rate corresponds to (CFU released by 0.1% DOC at T_{24}/CFU released by 0.1% DOC at T_0) × 100.

2.3. Molecular biology methods

Transformation: Electroporation was performed using a MicroPulser Electroporator (BioRad) following the parameters suggested by the manufacturer. Competent cells were prepared as described previously for *S. enterica* [13].

Deletion of the tet gene: The *tet* gene from plasmid pACYC184 was deleted by enzymatic restriction using the *Ava*I and *Cla*I sites. A fill-in reaction was done and the 2.8 kb vector was designated pSIF009 (pACYC184 Δ*tet*). pSIF009 transformed into *Salmonella* was used in macrophage infection assays.

Cloning of the tet gene: The *tet* gene was amplified by PCR from plasmid pACYC184 with primers tetF 5′-TGTAGCACCTGAAGTCAGCC-3′ and tetR 5′-GGAGTGGTGAATCCGTTAGC-3′ using Vent polymerase (New England Biolabs). The 1358 pb *tet* amplicon was cloned into the *Hin*cII site of pACYC177 to generate the plasmid pSIF010 (pACYC177::*tet*).

2.4. Gentamicin sensitivity assay

The susceptibility to the aminoglycoside antibiotic gentamicin was determined by minimal inhibitory concentration (MIC) assay. Strains were grown overnight and cultures were diluted in fresh LB medium to obtain 10^6 CFU ml^{-1}. Microwell plates were loaded with the 10^6 CFU ml^{-1} cultures and different concentrations of gentamicin ranging from 150 to 50 μg ml^{-1} were added to each well. Plates were incubated overnight and wells were evaluated for growth by spectrophotometry (OD_{630}). The MIC was considered the lowest drug concentration that reduced growth by more than 50% compared with growth in the control well.

2.5. Acid tolerance response

The acid tolerance response (ATR) response was determined by using a method based on that of Foster and Hall [14]. Briefly, overnight grown cultures were used to inoculate (1/100) fresh LB medium (pH 7.3) in duplicates and grown until OD_{600} was 0.2. The pH in one flask was then quickly adjusted to pH 5.5 with 1 N HCl for adaptation and culture were grown until OD_{600} was 0.4. At this stage the pH was adjusted to 3.3. Aliquots were withdrawn immediately (T_0) and then at 1 h intervals for 4 h (T_1–T_4). The number of resistant bacteria was determined as colony forming units (CFU) by plating on LB agar and, when necessary, on LB agar containing appropriate antibiotics.

2.6. Hydrogen peroxide sensitivity assay

The assay was performed as described by Boyer et al. [15]. Briefly, overnight grown cultures were used to inoculate (1/100) fresh LB medium without antibiotics, and the resulting cultures were incubated until the OD_{600} was 0.5. A 100 μl volume of each culture was mixed with 3 ml of molten top agar and poured onto an LB agar plate. A 7 mm-diameter Whatman filter disk impregnated with 10 μl of 30.4% hydrogen peroxide (H_2O_2) was placed in the center of the plate before incubation for 17 h at 37 °C. Alternatively, 400 mM H_2O_2 was added to overnight grown bacteria diluted to 10^7 cells ml^{-1} in PBS. Aliquots of bacteria were removed at timed intervals, diluted, and plated onto LB agar with or without antibiotics for quantisation of viable cells [16]. The assay was also performed using a lower bacterial density (5×10^5 cells ml^{-1}).

3. Results

3.1. Effect of commonly used plasmid vectors

We have investigated the effect of commonly used cloning vectors on the survival of *S. enterica* serovar *typhi* (*S. typhi*) following macrophage infection. Plasmids pACYC184, pACYC177, pWSK29, pBR322, pSC101 or pBeloBAC11 were introduced into *S. typhi* strain χ3744 by electoporation. These plasmids carried genes encoding resistance to different antibiotics (ampicillin, chloramphenicol, kanamycin, and tetracycline) and different origins of replication (p15A, pSC101, pMB1, OriS) (Table 1). Survival of *Salmonella* carrying either plasmid pACYC177, pWSK29 or pBeloBAC11 was similar to the bacteria without plasmid 24 h post-infection (Fig. 1). There was however, a significant ($p < 0.05$) decrease in survival of intracellular bacteria containing either plasmid pACYC184, pBR322 or pSC101 (Fig. 1). This decrease in bacterial survival rate was also observed when the murine macrophage cell line RAW267.4 was used (data not shown). A similar effect on bacterial survival rate was seen when *S. enterica* serovar *typhimurium* strain SR11 was used as the bacterial host for carrying these plasmids (data not shown). Decreased survival of bacteria in macrophages occurred when bacteria were grown either overnight standing (conditions that induce the *Salmonella* pathogenicity island I, encoding a locus for invasion), or grown to log phase with aeration (data not shown). All plasmids were stable during infection assays as determined by plating

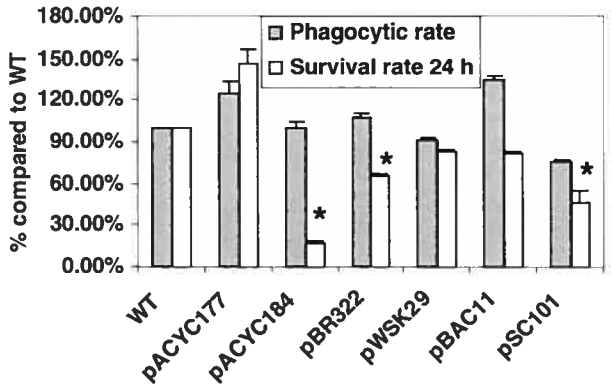

Fig. 1. Survival assays. Comparison of the abilities of the wild-type ISP1820 with or without plasmid to survive within THP-1 macrophage-like cells. For each strain at each time point, the number of bacteria recovered was expressed as a percentage of the number that were present in the inoculum. The values for percent recovery were normalized relative to that of the wild-type control (plasmid-free), which was designated 100% at each time point. The results represent an average of at least three distinct trials done in duplicate. Standard error bars are indicated. Strains carrying plasmid that demonstrated significant ($p < 0.05$) difference to the plasmid-free strain are indicated (*).

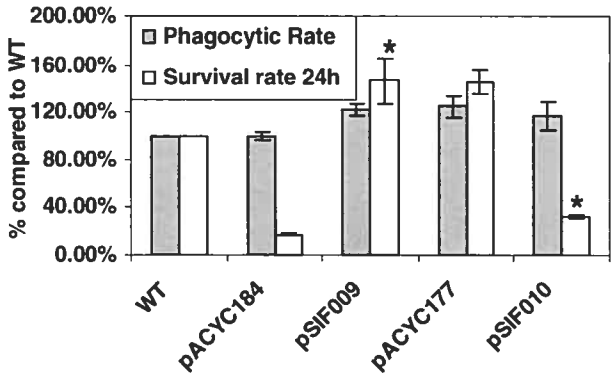

Fig. 2. Role of the *tet* gene during macrophage infection. Comparison of the abilities of bacteria carrying plasmid with or without the *tet* gene to survive within THP-1 macrophage-like cells. The results represent an average of at least three distinct trials done in duplicate. Standard error bars are indicated. Bacteria carrying plasmid that demonstrated a significant difference ($p < 0.05$) to the plasmid-free strain are indicated (*).

the bacteria following macrophage lysis on media with and without antibiotic selection (data not shown).

3.2. Effect of the tetracycline gene during macrophage infection

Plasmids pSC101, pACYC184 and pBR322 differ in copy number and belong to different incompatibility groups (Table 1). These plasmids all contain the tetracycline resistance gene of pSC101 [17–19]. In order to determine if the tetracycline resistance gene was responsible for the lower survival rate observed in bacterial cells carrying pACYC184, we performed macrophage interaction assays with *S. typhi* carrying a plasmid derived from pACYC184 but lacking the *tet* gene (pSIF009). The strain carrying pSIF009 (pACYC184 Δ*tet*) demonstrated a significant increase ($p < 0.05$) in survival rate compared to the strain carrying pACYC184, and the survival rate was similar to the plasmid-free wild-type strain. The intracellular survival rate of the strain carrying pSIF009 was also similar to survival of *S. typhi* containing the cloning vector pACYC177, which has the same origin of replication of pACYC184 (p15A origin) (Fig. 2). Plasmid pACYC177 was used to determine if the *tet* gene was responsible for the reduced survival rate of *S. typhi* cells carrying pACYC184 in macrophages. We cloned the *tet* gene into pACYC177 to obtain pSIF010 (pACYC177::*tet*). The survival rate of the bacteria harboring pSIF010 (pACYC177::*tet*) was significantly diminished ($p < 0.05$) as compared to those carrying pACYC177 or the plasmid-free strain (Fig. 2).

3.3. Growth profile

The growth rate of *S. typhi* cells carrying either plasmid pACYC177 or pACYC184, with or without the *tet* gene, was measured. In LB medium, the bacterial cells showed similar growth phenotypes. However, carriage of plasmid pACYC184 (with or without the *tet* gene) caused an initial lag in bacterial growth, by late log phase the defect was overcome and growth was equal to that of plasmid-free bacteria (Fig. 3). This phenotype was observed in presence or absence of selective pressure in the medium, and the same results were obtained when bacteria were grown in tissue culture medium (RPMI) or in minimal M9 medium supplemented with glucose or glycerol as carbon source.

3.4. In vitro stresses

Gentamicin is an aminoglycoside antibiotic that cannot effectively enter eukaryotic cells and it is commonly used during infection of macrophages to kill extracellu-

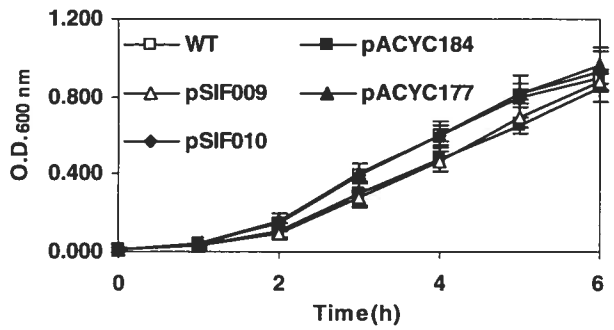

Fig. 3. Growth profile of strains carrying plasmid with or without the *tet* gene cultured in LB broth.

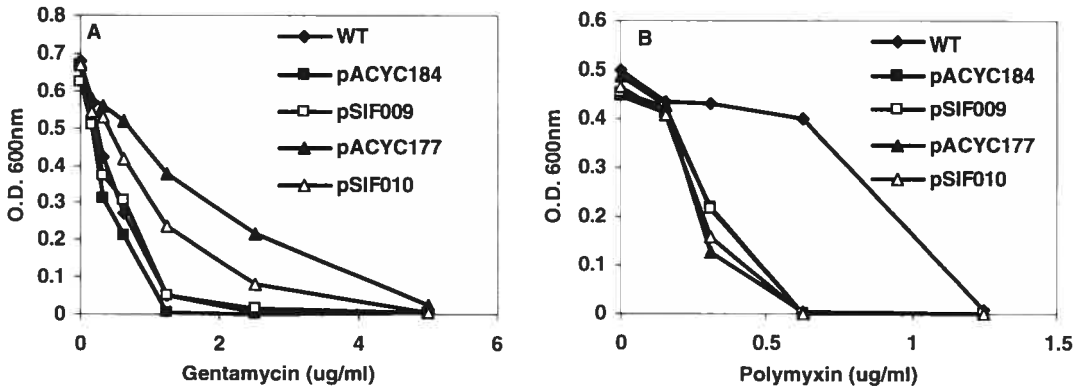

Fig. 4. Antibiotic susceptibility. Minimal inhibitory concentration (MIC) of (A) gentamicin or (B) polymyxin for strain carrying plasmid with or without the *tet* gene. The MIC for gentamicin of the strains carrying plasmid with the *tet* gene is significantly lower than the MIC of the strain carrying plasmid without the *tet* gene ($p < 0.05$).

lar bacteria. The TetA protein confers increased susceptibility to aminoglycoside antibiotics [20] making it necessary to test the susceptibility of the strains used in our study to gentamicin. Strains carrying plasmids containing the *tet* gene were more susceptible to killing by gentamicin than the strains carrying plasmid without *tet*. The MIC of the strains carrying plasmids containing *tet* was 2-fold lower than the strain carrying a plasmid without the *tet* gene ($p < 0.05$) (Fig. 4(A)). The MIC for wild-type strain was 0.625 $\mu g ml^{-1}$ The MIC for strain carrying pACYC184 was 0.312 $\mu g ml^{-1}$ compared to 0.625 $\mu g ml^{-1}$ for the strain carrying pACYC184 without *tet* (pSIF009). The MIC for strain carrying the plasmid pACYC177 with *tet* (pSIF010) was 1.25 $\mu g ml^{-1}$ compared to 2.5 $\mu g ml^{-1}$ for strain carrying pACYC177. The strains carrying plasmid pACYC177, with or without the *tet* gene, were more resistant to gentamicin than the wild-type strain or the other plasmid-bearing strains tested. Interestingly, during infection, the strain carrying the plasmid pACYC177 with the *tet* gene (pSIF010) had a significantly lower survival rate than the wild-type strain or strains carrying plasmid without the *tet* gene (Fig. 2). Thus, the lower level of survival in macrophages seen for strains containing the *tet* gene is not a result of increased susceptibility to gentamicin. Polymyxin B is another antibiotic that can be used to determine internalization and survival of intracellular bacteria. To further investigate the effect of the *tet* gene on survival of *S. typhi* in macrophages, we used polymyxin instead of gentamicin. The MIC to polymyxin of the all strains carrying plasmid, with or without the *tet* gene, was 0.312 $\mu g ml^{-1}$ (Fig. 4(B)). For the wild-type strain the MIC to polymyxin was 1.25 $\mu g ml^{-1}$ (Fig. 4(B)). When polymyxin was used to kill extracellular bacteria during infection of macrophages, a decrease in survival of intracellular bacteria containing plasmid with the *tet* gene similar to that seen when gentamicin was used was observed (Fig. 5).

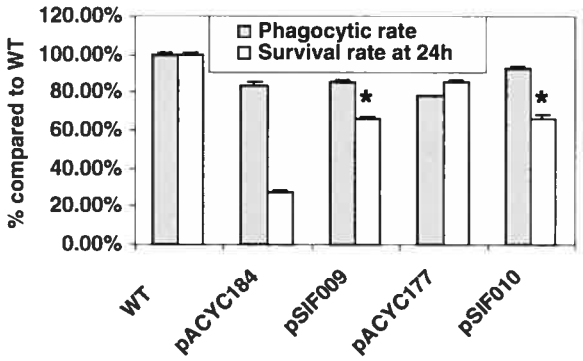

Fig. 5. Survival assays using polymyxin. Comparison of the abilities of wild-type strain ISP1820 with or without plasmid to survive within THP-1 macrophage-like cells. See Fig. 1 legend. Strains carrying plasmid that demonstrated significant ($p < 0.05$) difference to the plasmid-free strain are indicated (*).

ATR and resistance to H_2O_2 of plasmid-bearing strains (with or without *tet*) was tested to determined if such harsh physical conditions, which are produced within phagocytes, were responsible for the observed decrease of *Salmonella* survival. ATR was inducible and the level of acid resistant bacteria was similar in plasmid-free strains or strains bearing plasmids with or without the *tet* gene. All the stains demonstrated survival levels of 40% after 4 h of acid shock. For all strains an inhibition zone to hydrogen peroxide of approximately 38 mm was measured. Sensitivity to H_2O_2 was also assessed by adding 400 mM H_2O_2 directly to overnight grown bacteria diluted to 10^7 cell ml^{-1}. Strains carrying plasmids containing the *tet* gene were significantly ($p < 0.05$) more susceptible to H_2O_2 at 3 and 4 h after exposure than the wild-type strain or strains carrying plasmid without *tet* (Fig. 6). When a lower bacterial density of 5×10^5 cell ml^{-1} in stationary phase, which may be more similar to bacterial densities during infection of macrophages, was used no difference was

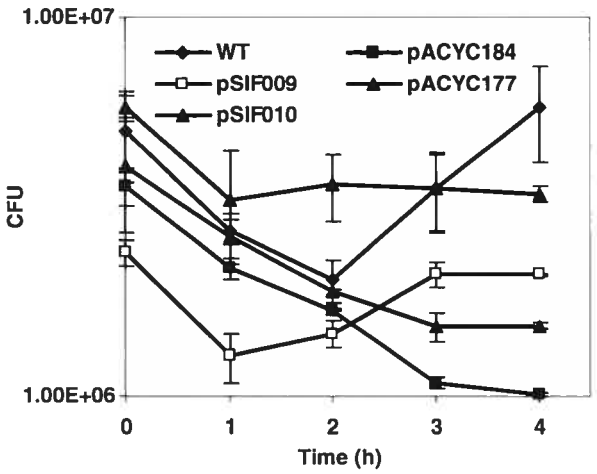

Fig. 6. Hydrogen peroxide sensitivity assay for strains cultured overnight carrying plasmids with or without the *tet* gene in presence of 400 μM of H_2O_2.

observed in susceptibility to H_2O_2 between the strains carrying the vector with or without the *tet* gene after 4 h of peroxide exposure (data not shown).

4. Discussion

Even in the post-genomic era, many genes still have unknown functions. Molecular genetics, including cloning mutagenesis and complementation, are necessary approaches to determine the role and function of genes. Cloning vectors such as pBR322 [17], pACYC184 [18] and pACYC177 [18] are useful not only in molecular genetic studies but also in biotechnology. The expression or inactivation of antibiotic resistance genes facilitates selection and counterselection of plasmid-containing bacteria carrying foreign DNA inserts.

In order to assign a function to a gene, the gene must be knocked out and then reintroduced to demonstrate that it is able to restore or complement a wild-type phenotype to the mutant. While complementing a mutant using plasmid pACYC184, we also cloned the empty vector into the wild-type strain as a control and found that the carriage of the vector itself affects the survival of *Salmonella* in macrophages. We then investigated the effect of different commonly used plasmid vectors on survival of *Salmonella* in macrophages. At 24 h post-infection, there was a significant 80% decrease in survival of intracellular bacteria containing plasmids pSC101 and pACYC184 as compared to plasmid-free bacteria. A similar reduction in the survival rate was also observed with bacteria bearing vector pBR322. The tetracycline resistance gene is a genetic element common to these vectors.

Tetracycline resistance is mediated by at least 30 distinct systems in different bacteria, [21] and four classes of plasmid-encoded *tet* genes (A, B, C, and D) have been described in Gram-negative bacteria [22]. These four classes encode related inner membrane Tet proteins that are distinguished by differences in amino acid sequences and the relative levels of resistance to tetracycline they confer [23]. The most studied of the four classes are the two inducible tetracycline resistance systems (i) *tetAR* (class B) encoded on transposon Tn*10* [24,25], and (ii) the *tet* gene from the low copy plasmid pSC101 (class C). Plasmids pACYC184 and pBR322 as well as other higher copy cloning vectors contain the *tet* gene from pSC101 [26]. Both native tetracycline resistance systems from Tn*10* and pSC101 only produce the tetracycline resistance proteins following induction by the presence of tetracycline [24–26]. However, the *tet* gene from pSC101 cloned into pBR322 and pACYC184 is constitutively expressed due to the absence of a repressor protein and/or a higher copy number of the *tet* gene [26,27]. *S. typhi* carrying pACYC184, pBR322, or pSC101 had decreased survival as compared to plasmid free *S. typhi* and *S. typhi* harboring plasmid pACYC177, pWSK29, or pBeloBAC11 (Fig. 1). pSC101 is a lower copy number plasmid than pACYC184 and pBR322, thus the fact that all three plasmids had a similar effect on bacterial survival in macrophages means that we can discard the hypothesis of the copy number or increased expression of the *tet* gene being responsible for the phenotype we observed.

In addition to tetracycline efflux, it was demonstrated that expression of the *tet* gene causes several pleiotropic effects. The TetA protein confers increased susceptibility to specific aminoglycoside antibiotics [20], heavy metals [28], and specific organic acids [29,30]. Further, TetA complements certain defects in potassium uptake [31] and increases the supercoiling of plasmid DNA harboring the *tet* gene [32]. In addition, overexpression of the *tet* gene has been shown to reduce cell viability [23–34]. This reduction in cell viability is gene dosage-dependent, and is exacerbated by increased expression of the *tet* gene [33]. The physiological basis of the pleiotropic effects of *tet* is not known. It is reasonable to assume that these effects are a direct consequence of the structural features of the Tet protein, and that the binding of additional Tet proteins to the inner membrane may interfere with some vital bacterial function.

As expected, strains carrying plasmid with the *tet* gene were more susceptible to gentamicin, as demonstrated by a lower MIC. Gentamicin is a aminoglygoside that is poorly interanalysed by eukaryotic cells, therefore the antibiotic selectively kills extracellular bacteria without affecting bacteria within host cells [35]. However, some reports have indicated that gentamicin enters macrophages and kills intracellular bacteria [36]. Thus, the decrease in survival of intracellular bacteria containing plasmid with the *tet* gene could be because of the entrance of gentamicin inside macrophages. In our study

this possibility was ruled out by using the antibiotic polymyxin B to kill extracellular bacteria during infection of macrophages. When polymyxin was used the decrease in survival rate observed in strains carrying plasmid with the *tet* gene was still observed and all the strains, with or without the *tet* gene, demonstrated a similar MIC to polymyxin.

In our hands, growth profiles of bacteria with or without plasmid in LB broth were similar, however a consistent growth defect for *Salmonella* strains bearing plasmids pACYC184 (with or without the *tet* gene) was observed. This growth defect was most likely due to a portion of the plasmid other then the *tet* gene as removal of the gene did not lead to wild-type growth, and addition of the *tet* gene to plasmid pACYC177 did not result in a growth defect. We have tested in vitro stresses that can mimic the macrophage environment such as acidity and the presence of hydrogen peroxide. Resistance to acid was similar in the plasmid-free strain or strains bearing plasmids with or without the *tet* gene. A difference in susceptibility to H_2O_2 was observed between strains carrying plasmids with the *tet* gene and the wild-type strain or strains carrying plasmids without the *tet* gene at bacterial densities of 1×10^7 cells ml^{-1} but not at lower densities that correlated to the bacterial density during infection. The increased susceptibility to H_2O_2 seen at high bacterial densities could represent a pleiotropic effect of the *tet* gene. The THP-1 macrophages has a basal level production of H_2O_2 that is approximately 25 picomol 10^6 cells [37]. However, the correlation between the increased in sensitivity to H_2O_2 and the lower survival of bacteria within macrophages needed to be further investigated.

Diaz-Ricci et al. [5] have demonstrated that plasmid maintenance enhances gene expression, independently of the host, type and number of plasmids, including pACYC184 and pBR322. An increase of intracellular cyclic AMP in cells carrying plasmids was observed and higher β-galactosidase activity was measured. They ruled out any antibiotic-mediated gene expression enhancement by control experiments in which different antibiotics were used. They determined that the effect was due to plasmid maintenance and that antibiotics have no significant influence on gene expression. In our model, the mammalian host cell and culture medium lack the selective pressure(s) for plasmid encoded resistance genes, demonstrating that it was the presence of the *tet* gene in the plasmid and not exogenous antibiotics that caused the observed defect in bacterial survival rates.

Multicopy plasmids have greatly helped gene structure–function studies. However, the use of such plasmids can lead to copy number artifacts. Furthermore, as demonstrated in our current study, the presence of the tetracycline resistance gene should also be considered. The re-introduction of the mutated gene at wild-type levels would be the ideal solution for gene complementation. Several methods have been developed to insert a stable copy of a gene onto bacterial chromosomes allowing for the study of their function in single copies without the need for antibiotic resistance markers [38–41]. This can be applicable for complementation, where a single copy of the mutated gene can be stably inserted into the chromosome of the mutated strain. Thus pleiotropic effects from plasmid maintenance genes can be avoided in this system and the use of selective agents, such as antibiotics, is no longer necessary. Use of the tetracycline resistance gene for mutation or complementation should be carefully considered in light of our finding that the presence of the gene can effect bacterial survival and thus bias results.

Acknowledgements

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References

[1] Bailey, J.E., Da Silva, N.A., Peretti, S.W., Seo, J.H. and Srienc, F. (1986) Studies of host–plasmid interactions in recombinant microorganisms. Ann. N.Y. Acad. Sci. 469, 194–211.

[2] Birnbaum, S. and Bailey, J.E. (1991) Plasmid presence changes the relative levels of many host cell proteins and ribosome components in recombinant *Escherichia coli*. Biotechnol. Bioeng. 37, 736–745.

[3] Brownlie, L., Stephenson, J.R. and Cole, J.A. (1990) Effect of growth rate on plasmid maintenance by *Escherichia coli* HB101(pAT153). J. Gen. Microbiol. 136, 2471–2480.

[4] Mason, C.A. and Bailey, J.E. (1989) Effect of plasmid presence on growth and enzyme activity of *Escherichia coli* DH5a. Appl. Microbiol. Biotechnol. 32, 54–60.

[5] Diaz-Ricci, J.C., Bode, J., Rhee, J.I. and Schugerl, K. (1995) Gene expression enhancement due to plasmid maintenance. J. Bacteriol. 177, 6684–6687.

[6] Lenski, R.E. and Bouma, J.E. (1987) Effects of segregation and selection on instability of plasmid pACYC184 in *Escherichia coli* B. J. Bacteriol. 169, 5314–5316.

[7] Godwin, D. and Slater, J.H. (1979) The influence of the growth environment on the stability of a drug resistance plasmid in *Escherichia coli* K12. J. Gen. Microbiol. 111, 201–210.

[8] Helling, R.B., Kinney, T. and Adams, J. (1981) The maintenance of plasmid-containing organisms in populations of *Escherichia coli*. J. Gen. Microbiol. 123, 129–141.

[9] Noack, D., Roth, M., Geuther, R., Muller, G., Undisz, K., Hoffmeier, C. and Gaspar, S. (1981) Maintenance and genetic stability of vector plasmids pBR322 and pBR325 in *Escherichia coli* K12 strains grown in a chemostat. Mol. Gen. Genet. 184, 121–124.

[10] Jones, I.M., Primrose, S.B., Robinson, A. and Ellwood, D.C. (1980) Maintenance of some ColE1-type plasmids in chemostat culture. Mol. Gen. Genet. 180, 579–584.

[11] Finlay, B.B. and Brumell, J.H. (2000) *Salmonella* interactions with host cells: in vitro to in vivo. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 355, 623–631.
[12] Ralph, P. and Nakoinz, I. (1977) Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS. J. Immunol. 119, 950–954.
[13] O'Callaghan, D. and Charbit, A. (1990) High efficiency transformation of *Salmonella typhimurium* and *Salmonella typhi* by electroporation. Mol. Gen. Genet. 223, 156–158.
[14] Foster, J.W. and Hall, H.K. (1990) Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. 172, 771–778.
[15] Boyer, E., Bergevin, I., Malo, D., Gros, P. and Cellier, M.F. (2002) Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica* serovar Typhimurium. Infect. Immun. 70, 6032–6042.
[16] Buchmeier, N.A., Libby, S.J., Xu, Y., Loewen, P.C., Switala, J., Guiney, D.G. and Fang, F.C. (1995) DNA repair is more important than catalase for *Salmonella* virulence in mice. J. Clin. Invest. 95, 1047–1053.
[17] Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L. and Boyer, H.W. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2, 95–113.
[18] Chang, A.C. and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134, 1141–1156.
[19] Chang, A.C. and Cohen, S.N. (1974) Genome construction between bacterial species in vitro: replication and expression of *Staphylococcus* plasmid genes in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 71, 1030–1034.
[20] Griffith, J.K., Kogoma, T., Corvo, D.L., Anderson, W.L. and Kazim, A.L. (1988) An N-terminal domain of the tetracycline resistance protein increases susceptibility to aminoglycosides and complements potassium uptake defects in *Escherichia coli*. J. Bacteriol. 170, 598–604.
[21] Levy, S.B., McMurry, L.M., Barbosa, T.M., Burdett, V., Courvalin, P., Hillen, W., Roberts, M.C., Rood, J.I. and Taylor, D.E. (1999) Nomenclature for new tetracycline resistance determinants. Antimicrob. Agents. Chemother. 43, 1523–1524.
[22] McMurry, L., Petrucci Jr., R.E. and Levy, S.B. (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 77, 3974–3977.
[23] Levy, S. (1984) Resistance of the tetracyclines In: Antimicrobial Drug Resistance (Bryan, L., Ed.), pp. 191–240. Academic Press Inc, New York.
[24] Daniels, D.W. and Bertrand, K.P. (1985) Promoter mutations affecting divergent transcription in the Tn*10* tetracycline resistance determinant. J. Mol. Biol. 184, 599–610.
[25] Kleckner, N. (1989) Transposon Tn*10* In: Mobile DNA (Berg, D.E. and Howe, M.M., Eds.), pp. 227–268. ASM Press, Washington DC.
[26] Backman, K. and Boyer, H.W. (1983) Tetracycline resistance determined by pBR322 is mediated by one polypeptide. Gene 26, 197–203.
[27] Lenski, R.E., Simpson, S.C. and Nguyen, T.T. (1994) Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. J. Bacteriol. 176, 3140–3147.
[28] Griffith, J.K., Buckingham, J.M., Hanners, J.L., Hildebrand, C.E. and Walters, R.A. (1982) Plasmid-conferred tetracycline resistance confers collateral cadmium sensitivity of *E. coli* cells. Plasmid 8, 86–88.
[29] Bochner, B.R., Huang, H.C., Schieven, G.L. and Ames, B.N. (1980) Positive selection for loss of tetracycline resistance. J. Bacteriol. 143, 926–933.
[30] Herrin Jr., G.L., Russell, D.R. and Bennett, G.N. (1982) A stable derivative of pBR322 conferring increased tetracycline resistance and increased sensitivity to fusaric acid. Plasmid 7, 290–293.
[31] Dosch, D.C., Salvacion, F.F. and Epstein, W. (1984) Tetracycline resistance element of pBR322 mediates potassium transport. J. Bacteriol. 160, 1188–1190.
[32] Lodge, J.K., Kazic, T. and Berg, D.E. (1989) Formation of supercoiling domains in plasmid pBR322. J. Bacteriol. 171, 2181–2187.
[33] Valenzuela, M.S., Ikpeazu, E.V. and Siddiqui, K.A. (1996) *E. coli* growth inhibition by a high copy number derivative of plasmid pBR322. Biochem. Biophys. Res. Commun. 219, 876–883.
[34] Eckert, B. and Beck, C.F. (1989) Overproduction of transposon Tn*10*-encoded tetracycline resistance protein results in cell death and loss of membrane potential. J. Bacteriol. 171, 3557–3559.
[35] Vaudaux, P. and Waldvogel, F.A. (1979) Gentamicin antibacterial activity in the presence of human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. 16, 743–749.
[36] Drevets, D.A., Canono, B.P., Leenen, P.J. and Campbell, P.A. (1994) Gentamicin kills intracellular *Listeria monocytogenes*. Infect. Immun. 62, 2222–2228.
[37] Mouithys-Mickalad, A., Deby-Dupont, G., Nys, M., Lamy, M. and Deby, C. (2001) Oxidative processes in human promonocytic cells (THP-1) after differentiation into macrophages by incubation with *Chlamydia pneumoniae* extracts. Biochem. Biophys. Res. Commun. 287, 781–788.
[38] Boyd, D., Weiss, D.S., Chen, J.C. and Beckwith, J. (2000) Towards single-copy gene expression systems making gene cloning physiologically relevant: lambda InCh, a simple *Escherichia coli* plasmid-chromosome shuttle system. J. Bacteriol. 182, 842–847.
[39] Huang, L.C., Wood, E.A. and Cox, M.M. (1997) Convenient and reversible site-specific targeting of exogenous DNA into a bacterial chromosome by use of the FLP recombinase: the FLIRT system. J. Bacteriol. 179, 6076–6083.
[40] Martinez-Morales, F., Borges, A.C., Martinez, A., Shanmugam, K.T. and Ingram, L.O. (1999) Chromosomal integration of heterologous DNA in *Escherichia coli* with precise removal of markers and replicons used during construction. J. Bacteriol. 181, 7143–7148.
[41] Haldimann, A. and Wanner, B.L. (2001) Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure–function studies of bacteria. J. Bacteriol. 183, 6384–6393.
[42] Shizuya, H., Birren, B., Kim, U.J., Mancino, V., Slepak, T., Tachiiri, Y. and Simon, M. (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. USA 89, 8794–8797.
[43] Wang, R.F. and Kushner, S.R. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene 100, 195–199.

Une bactérie au pays des biopuces : étude du transcriptome de *Salmonella* dans des macrophages infectés

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> *Salmonella enterica* est une bactérie pathogène causant une variété de maladies chez l'homme et l'animal. Spécifique à l'homme, le serovar Typhi est l'agent étiologique de la fièvre typhoïde, une infection systémique dont on évalue les ravages à quelque 16 millions de nouveaux cas et à plus de 600 000 morts par année. La crainte de travailler avec cette bactérie très pathogène et l'absence d'un modèle animal adéquat compliquent l'étude des mécanismes de virulence de Typhi. En fait, la majorité des connaissances sur Typhi proviennent d'extrapolations à partir d'études utilisant Typhimurium, un serovar apparenté, dans un modèle murin de fièvre typhoïde.

La survie de *Salmonella* dans les macrophages est conditionnelle à l'expression de plusieurs gènes qu'il importe d'identifier pour prévenir et guérir les infections causées par cette bactérie. Les biopuces sont couramment utilisées pour déterminer le profil global d'expression génique dans des conditions qui miment l'infection. Cependant les limites intrinsèques de cette approche ne permettent pas l'identification des gènes bactériens exprimés *in vivo*. Les facteurs limitants comprennent la faible quantité d'ARN bactérien et la courte demi-vie de l'ARNm non polyadénylé qui, de plus, se retrouve mélangé avec l'ARNr et l'ARN provenant de l'hôte. Quant à elle, la technique SCOTS (*selective capture of transcribed sequences*) permet l'identification de gènes bactériens exprimés dans les cellules de l'hôte par clonage et séquençage des transcrits [1]. Nous avons récemment réussi à combiner les qualités de ces deux approches [2]. En effet, les transcrits bactériens obtenus par la technique SCOTS ont été utilisés en conjonction avec les biopuces, ce qui nous a permis d'obtenir le profil global d'expression génique de la bactérie à partir de macrophages humains infectés [2].

Grâce à la technique SCOTS, l'ARN total de macrophages humains THP-1 infectés a été isolé et converti en ADNc contenant les amorces spécifiques par transcriptase inverse. Cet ADNc initial contient un mélange correspondant à l'ARN eucaryote et procaryote. L'ADNc bactérien de ce mélange a été capturé par hybridation avec l'ADN génomique bactérien et ensuite amplifié grâce aux amorces spécifiques. Dans notre cas, trois cycles de capture ont été effectués. L'ADNc provenant de chacune de ces étapes a été marqué et utilisé pour l'hybrider à des biopuces de *Salmonella* [3]. Nos résultats indiquent qu'une fai-

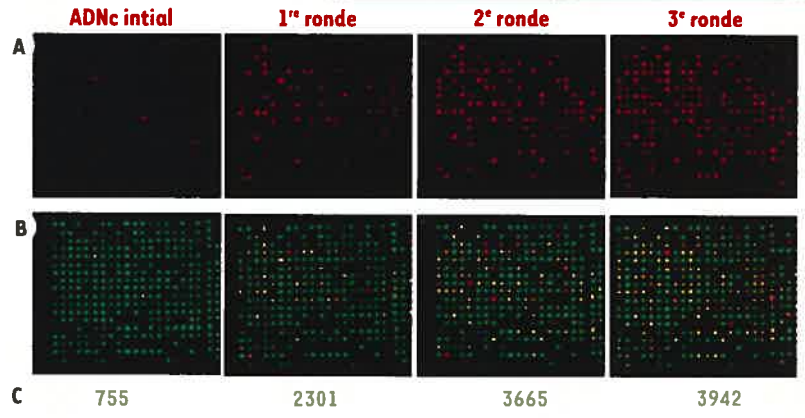

Figure 1. *Reproduction d'une portion des biopuces hybridées avec l'ADNc initial ou l'ADNc capture avec SCOTS, provenant de macrophages infectés 2 heures après la phagocytose (T2). A.* ADNc seulement marqué avec le fluorochrome Cy5 (rouge). *B.* ADNc et ADN génomique marqué avec le fluorochrome Cy3 (vert). *C.* Nombre de gènes détectés (signal plus grand que celui du bruit de fond).

ble proportion des transcrits provenant de l'ADNc initial présentent un signal assez fort pour être détectés *(Figure 1)*. Ces résultats démontrent le peu de sensibilité obtenu lorsque l'ADNc initial est utilisé et confirment les limites d'identification directe de transcrits bactériens durant l'infection. En revanche, le nombre de gènes détectés augmente avec les cycles successifs de la méthode SCOTS *(Figure 1)*. Ainsi, nous avons confirmé que la complexité de l'ADNc obtenu par la technique SCOTS était suffisante pour obtenir le transcriptome bactérien *in vivo*.

Nous avons donc utilisé la technique SCOTS pour obtenir de l'ADNc de Typhi à partir du surnageant de macrophages infectés, après la phagocytose (T0), à 2h (T2), 8h (T8) et 24h (T24) post-infection [2]. Nous avons mis en évidence une différence d'expression significative de 36 % du génome de Typhi, la répression de 138 gènes et l'induction de 117 gènes, comparés au surnageant de culture. Parmi les gènes induits, plusieurs facteurs de virulence connus ont été identifiés, corroborant nos résultats, ainsi que plusieurs gènes aux fonctions inconnues, qui pourraient correspondre à de nouveaux facteurs de virulence. Les résultats de biopuces ont été confirmés en quantifiant l'expression de certains gènes par PCR quantitatif en temps réel [4].

Le chromosome de *Salmonella* possède plusieurs insertions de larges régions d'ADN qui contiennent des gènes de virulence que l'on nomme îlots de pathogénicité (SPI). Les SPIs jouent un rôle clé dans la pathogenèse de *Salmonella*. SPI-1 et SPI-2 codent pour deux systèmes de sécrétion de type trois ayant des rôles différents : SPI-1 participe à l'invasion tandis que SPI-2 contribue à la survie intracellulaire [5]. Chez Typhi, les gènes de SPI-1 ont été identifiés comme étant induits au début de l'infection (T0) et, par la suite, réprimés pour le reste de l'infection *(Figure 2)*. Les gènes de SPI-2 sont induits au début de l'infection, ainsi que pour toute la durée de l'infection *(Figure 2)*. Ces résultats concordent avec le rôle biologique de ces SPI et valident nos résultats.

De plus, Typhi répond fortement aux peptides antimicrobiens (PA) présents à l'intérieur de la vacuole des macrophages en induisant les gènes de résistance qui modifient les lipopolysaccharides de la membrane bactérienne *(Figure 2)*. Les gènes participant à la mobilité, ainsi qu'au transport du fer, un élément essentiel à la croissance des bactéries, sont réprimés chez Typhi lors de l'infection, suggérant que le fer est disponible dans la vacuole *(Figure 2)*. Il n'y a pas eu de différence dans l'expression des gènes prenant part au stress oxydatif *(Figure 2)*. La comparaison des transcriptomes de Typhi et Typhimurium (récemment publié [6]) a révélé certaines différences. L'induction constitutive du régulateur transcriptionnel de gènes de virulence PhoP est observée chez Typhi durant l'infection ; en revanche, il n'y a pas de différence d'expression chez Typhimurium. Contrairement à Typhimurium qui utilise le gluconate comme source de carbone, Typhi semble plutôt se servir des acides gras. Effectivement, le gène *aceA* codant pour l'isocitrate lyase, une enzyme permettant l'utilisation des acides gras *via* le cycle du glyoxylate, est induit à partir de 8 h post-infection, tandis qu'il n'est pas induit chez Typhimurium 12 h post-infection.

En conclusion, notre étude a démontré que l'utilisation de la technique SCOTS et des biopuces permet d'obtenir le transcriptome d'une bactérie intracellulaire dans des conditions normales d'infection. Le transcriptome de Typhi à partir de macrophages infectés a ainsi été obtenu et manifeste un profil attendu pour les gènes de virulence. La caractérisation de certains gènes inconnus exprimés durant l'infection sera importante pour élucider les mécanismes de pathogénie de Typhi, ouvrant la voie au développement d'une meilleure approche préventive et thérapeutique. ◊

Salmonella transcriptome during macrophage infection

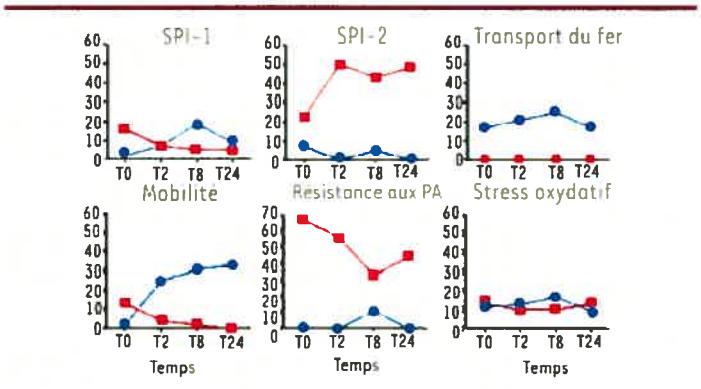

Figure 2. *Analyse de l'expression de gènes regroupés selon leur fonction.* Nombre de gènes (%), dans chacune des classes, qui sont significativement induits (courbe rouge) ou réprimés (courbe bleue) ($p < 0,001$, changement d'expression de plus de 2 fois par rapport au surnageant). PA : peptides antimicrobiens.

RÉFÉRENCES

1. Daigle F, Hou JY, Clark-Curtiss JE. Microbial gene expression elucidated by selective capture of transcribed sequences (SCOTS). *Meth Enzymol* 2002 ; 358 : 108-22.
2. Faucher SP, Porwollik S, Dozois CM, *et al.* Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. *Proc Natl Acad Sci USA* 2006 ; 103 : 1906-11.
3. Porwollik S, Frye J, Florea LD, *et al.* A non-redundant microarray of genes for two related bacteria. *Nucleic Acids Res* 2003 ; 31 : 1869-76.
4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 2001 ; 25 : 402-8.
5. Hensel M. Evolution of pathogenicity islands of *Salmonella enterica*. *Int J Med Microbiol* 2004 ; 294 : 95-102.
6. Eriksson S, Lucchini S, Thompson A, *et al.* Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* 2003 ; 47 : 103-18.