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Interaction d'*Escherichia coli* entérohémorragique (EHEC) avec *Acanthamoeba castellanii* et rôle du régulon Pho chez les EHEC

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INTERACTION D'*ESCHERICHIA COLI* ENTÉROHÉMORRAGIQUE (EHEC) AVEC *ACANTHAMOEBA CASTELLANII* ET RÔLE DU RÉGULON PHO CHEZ LES EHEC

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Résumé

Les EHEC de sérotype O157:H7 sont des agents zoonotiques d'origine alimentaire ou hydrique. Ce sont des pathogènes émergeants qui causent chez l'humain des épidémies de gastro-entérite aiguë et parfois un syndrome hémolytique-urémique. Les EHEC réussissent leur transmission à l'humain à partir de leur portage commensal chez l'animal en passant par l'étape de survie dans l'environnement. L'endosymbiose microbienne est une des stratégies utilisées par les bactéries pathogènes pour survivre dans les environnements aquatiques. Les amibes sont des protozoaires vivants dans divers écosystèmes et connus pour abriter plusieurs agents pathogènes. Ainsi, les amibes contribueraient à transmettre les EHEC à l'humain. La première partie de mon projet de thèse est centrée sur l'interaction de l'amibe *Acanthamoeba castellanii* avec les EHEC. Les résultats montrent que la présence de cette amibe prolonge la persistance des EHEC, et ces dernières survivent à leur phagocytose par les amibes. Ces résultats démontrent le potentiel réel des amibes à héberger les EHEC et à contribuer à leur transmission. Cependant, l'absence de Shiga toxines améliore leur taux de survie intra-amibe. Par ailleurs, les Shiga toxines sont partiellement responsables de l'intoxication des amibes par les EHEC. Cette implication des Shiga toxines dans le taux de survie intracellulaire et dans la mortalité des amibes démontre l'intérêt d'utiliser les amibes comme modèle d'interaction hôte/pathogène pour étudier la pathogénicité des EHEC.

Durant leur cycle de transmission, les EHEC rencontrent des carences en phosphate inorganique (Pi) dans l'environnement. En utilisant conjointement le système à deux composantes (TCS) PhoB-R et le système Pst (transport spécifique de Pi), les EHEC détectent et répondent à cette variation en Pi en activant le régulon Pho. La relation entre la virulence des EHEC, le PhoB-R-Pst et/ou le Pi environnemental demeure inconnue. La seconde partie de mon projet explore le rôle du régulon Pho (répondant à un stress nutritif de limitation en Pi) dans la virulence des EHEC. L'analyse transcriptomique montre que les EHEC répondent à la carence de Pi par une réaction complexe impliquant non seulement un remodelage du métabolisme général, qui est critique pour sa survie, mais aussi en coordonnant sa réponse de virulence. Dans ces conditions le régulateur PhoB contrôle directement l'expression des gènes du LEE et de l'opéron *stx2AB*. Ceci est confirmé par l'augmentation de la sécrétion de l'effecteur EspB et de la production et sécrétion de Stx2 en carence en Pi. Par ailleurs,

l'activation du régulon Pho augmente la formation de biofilm et réduit la motilité chez les EHEC. Ceci corrèle avec l'induction des gènes régulant la production de curli et la répression de la voie de production d'indole et de biosynthèse du flagelle et du PGA (Polymère β-1,6-N-acétyle-D-glucosamine).

Mots-clés: EHEC, phosphate environnemental, régulon Pho, Shiga toxine, SST3, *A. castellanii*

Abstract

EHEC O157:H7 are an emerging zoonotic food- and water-borne hazard highly pathogenic to humans and associated with diseases ranging from acute gastroenteritis to hemolytic uremic syndrome. From their commensal carriage by farm animals to human targets, EHEC pass through a crucial step of persistence in the open environment. Microbial endosymbiosis is one strategy used by pathogenic bacteria to survive in aquatic environments. Amoebae species are free-living protozoa found in diverse environmental habitats and known to host several water-borne pathogens. Thus amoebae could contribute to transmission of EHEC to humans. The first part of my PhD project was focused on interaction of the free-living amoebae *Acanthamoeba castellanii* with EHEC. The results showed that the presence of amoeba extends the persistence of EHEC that survived phagocytosis by amoebae. This demonstrates the real potential of amoebae to harbourd EHEC that may contribute to their transmission. However, absence of shiga toxins enhanced the intra-amoeba survival. Moreover, EHEC had a toxic and lethal effect on amoebae partially due to shiga toxins. The involvement of shiga toxins in the intracellular survival and mortality of amoebae suggests the value of using amoebae as a model of host/pathogen interactions to study the pathogenicity of EHEC.

During their transmission cycle, EHEC encounter limitation inorganic phosphate (Pi) in the environment. Using jointly the PhoB-R two-component system (TCS) and the Pst (Pi specific transport) system, EHEC detect and respond to this Pi limitation by activating the Pho regulon. The interplay between the EHEC virulence, the Pho-Pst and/or the environmental Pi remains unknown. The second part of my project explored the role of Pho regulon (responding to Pi-limitation stress) in the virulence of EHEC. Transcriptomic analysis showed that EHEC has evolved a sophisticated response to Pi deficiency involving not only biochemical strategies that are likely critical to its survival, but also coordinating its virulence response. In these conditions, the regulator PhoB regulates directly the expression of LEE and Stx2 genes. This is confirmed by an increase in EspB secretion and Stx2 production and secretion in low Pi conditions. Moreover, the activation of Pho regulon increases biofilm formation and reduces motility in EHEC. This correlated with the induction of genes regulating curli production and repression of indole production pathway and the flagellum and PGA biosynthesis.

Keywords: EHEC, environmental Phosphate, Pho regulon, Shiga toxin, T3SS, *A. castellanii*

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Discussion générale

Annexe # 1

Abréviations

Akt: Protein Kinase B

A/E: Attachant-effaçant

AAF: Fimbriae d'adhérence aggrégative

AcM: Anticorps monoclonal

AIEC: *E. coli* adhérentes-invasives

APEC: *E. coli* pathogène aviaire

ATCC: American Type Culture Collection

BFP: Pilus formant un empaquetage «bundle»

CDC: Centers for Disease Control and Prevention

Ces: Chaperone of *E. coli* secretion

CFA: Facteur antigénique de colonisation

COG: Cluster de gène orthologues

DAEC: *E. coli* adhérentes diffuses

DAF: Facteur decay- accélérant

EAEC: *E. coli* entéroaggrégatives

EAST1: Enteroaggregative *E. coli* heat-stable enterotoxin 1

EHEC: *E. coli* entérohémorragiques

EPEC: *E. coli* entéropathogènes

EIEC: *E. coli* entéroinvasives

EMSA: Test de retard de migration électrophorétique

EPS: Exopolysaccharides

Esc: *Escherichia* secretion component

Esp: *Escherichia* secretion protein

ETEC: *E. coli* entérotoxigéniques

ExPEC: *E. coli* pathogéniques extra-intestinales

FAK: kinase d'adhésion focale

Gb3: Récepteur globotriaosylceramide

GFP: Protéine à fluorescence verte.

HeLa: Cellules de lignée HeLa (Henrietta Lacks)

LB: Milieu Luria-Bertani

LEE: Locus d'effacement des entérocytes

Ler: Régulateur global du LEE

LPF: Long polar fimbriae

LPS: Lipopolysaccharides

Map: Protéine associée à la mitochondrie

MOPS: 3-Nmorpholino propanesulfonic acid

Nle: Effecteur non-LEE

OIs: O Islands

ORF: Cadre ouvert de lecture (open reading frame)

PAI: Îlot de pathogénicité

PCR: Polymerase chain reaction

PGA: Polymère β-1,6-N-acétyle-D-glucosamine

Pi: Phosphate inorganique

Pst : Système transport spécifique de Pi

PTT: Purpura thrombocytopénique

PYG: Peptone yeast glucose

QRT-PCR: Réaction de PCR quantitative en transcription réverse

RNAP: ARN polymérase

SDS-PAGE: Électrophorèse sur gel de polyacrylamide en sodium dodecyl sulfate

Sep: Secretion of *E. coli* proteins

ShET1: Entérotoxine 1 de *Shigella*

SHU: Syndrome hémolytique-urémique

SST3: Système de sécrétion de type 3 (T3SS)

STEC: *E. coli* producteur de Shiga toxines

Stx: Shiga toxines

TCS: Système à deux composantes

TEM: Microscopie électronique à transmission

UPEC: *E. coli* uropathogéniques

VBNC: Viable et non-cultivable

Zot : zonula occludens toxin

Dédicace

*À **Aude Demengeon**.*

Je te dédie cet ouvrage pour te témoigner mes sentiments et ma reconnaissance pour avoir cru en moi et m'avoir encouragé de réessayer une 2ème fois d'aller au bout de mon rêve.

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Introduction générale

Escherichia coli (*E. coli*) est un colibacille à Gram-négatif, anaérobie facultatif, découvert en 1885 en Allemagne par Théodore Escherich dans les selles de nourrissons [1]. C'est un commensal qui colonise la flore intestinale de l'homme et des animaux à sang chaud dès les premières heures suivant la naissance [2]. Généralement, la plupart des souches d'*E. coli* sont inoffensives et coexistent avec l'hôte dans le tractus intestinal. Ces souches d'*E. coli* peuvent être bénéfiques à l'hôte en la protégeant contre l'infection par des bactéries pathogènes [3] et en synthétisant la vitamine K dans l'intestin de l'hôte [4]. Cependant, certaines souches d'*E. coli* peuvent causer des maladies en influençant l'immunité de l'hôte ou en acquérant des caractères de virulence [5]. Parmi ces souches d'*E. coli* pathogènes, les EHEC font partie du groupe d'*E. coli* producteur de Shiga toxines (STEC) qui est particulièrement dangereux entrainant des conséquences sanitaires et économique désastreuses.

Les éclosions d'infections entériques causées par bactéries pathogènes restent encore un problème pour les pays industrialisés ou en voie de développement [6]. Ces éclosions d'infection ont un impact sur la santé humaine, spécialement chez les enfants, les personnes âgées et les immunodéprimés. L'impact économique est lié au coût élevé de la prise en charge des malades et aussi à la détérioration de la valeur des aliments contaminés et d'origine animale. La plupart des éclosions reportées sont associées à des bactéries pathogènes qui causent des affections intestinales et systémiques et qui sont d'origines alimentaires ou transmise par consommation d'eau potable. Parmi les bactéries pathogènes incriminées, les EHEC sont émergentes en santé publique et sont connues pour être associées à de nombreuses épidémies de diarrhée aqueuses et sanglantes et sont parfois mortelles. Malgré les efforts des services de santé publique et salubrité alimentaire pour les éradiquer et prévenir d'éventuelles éclosions d'épidémie, la mesure du risque et la récente épidémie de STEC O104:H4 en 2011 en Europe montrent que le danger persiste [7].

Durant leur cycle de vie, ces pathogènes peuvent subir les variations de 2 facteurs extrinsèques; i) La variation de l'hôte. Entre leur portage commensal chez les bovins et leur hôte définitive humain, les EHEC pourraient s'associer et/ou survivre dans les protozoaires omniprésents dans l'écosystème, ce qui favoriserait leur persistance et renforcerait leur

transmission et pouvoir infectieux. ii) La variation d'apports nutritifs de l'environnement qui parfois en cas de carence nutritive obligent ces bactéries à survivre et s'adapter à ces changements. Le phosphate est un des facteurs nutritifs essentiels auquel tout pathogène est sensible. En réponse à la carence en phosphate, le régulon Pho est induit chez les EHEC pour maintenir l'homéostasie en phosphate. Ce changement génotypique pourrait avoir des conséquences sur l'expression des gènes de virulence des EHEC et par conséquent modifier leur virulence dans ces conditions.

Chapitre I : Revue de littérature

1. Les *E. coli* entérohémorragiques (EHEC) de sérotype O157:H7

1.1. Le pathovar EHEC

Plusieurs *E. coli* sont considérées comme pathogènes émergents et sont impliqués chez l'humain dans des infections intestinales aiguës et parfois conduisant à un syndrome hémolytique-urémique (SHU) [8,9]. Sept catégories d' *E. coli* pathogènes ont été décrites: *E. coli* entéropathogènes (EPEC), *E. coli* entérohémorragiques (EHEC), *E. coli* entéroaggrégatives (EAEC), *E. coli* entérotoxigéniques (ETEC), *E. coli* à adhérence diffuse (DAEC), *E. coli* adhérentes invasives (AIEC) et *E. coli* entéroinvasives (EIEC) [5,10]. Bien que ces 7 pathovars se distinguent par leurs génomes, ils montrent des similitudes pour certains mécanismes pathogéniques et parfois peuvent entrainer des effets cliniques similaires [9] (Fig. 1). En effet, malgré la diversité des affections provoquées par les souches pathogènes d'*E. coli*, toutes ces souches utilisent une stratégie classique d'infection commune à d'autres agents pathogènes, dont les points clés sont: colonisation de la muqueuse, multiplication, évasion des défenses de l'hôte, puis les dommages à l'hôte.

Chaque type d'*E. coli* diarrhéogènes a ses propres caractéristiques uniques d'interaction avec les cellules eucaryotes. L'adhérence et colonisation ou invasion des cellules cibles pour ces sept catégories d'*E. coli* pathogènes (représentées par des couleurs différentes) sont présentés ci-dessous.

- EPEC (en jaune) adhérent aux entérocytes de l'intestin grêle et détruit la frontière formée par les microvillosités, causant des lésions typiques attachant effaçant (A/E). La désorganisation du cytosquelette est accompagnée par la diarrhée et une réponse inflammatoire. Trois caractéristiques sont identifiés: i. Adhésion initiale; ii. Translocation de protéines par le SST3; iii. Formation en piédestal.
- De manière similaire, EHEC (en rose) causent des lésions A/E, mais surtout au niveau du colon. Les EHEC se distinguent par la caractéristique de production de Stx. L'absorption systémique de Stx induit des complications potentiellement mortelles.
- ETEC (en orange) utilisent des facteurs de colonisation (CFs) pour adhérer aux entérocytes de l'intestin grêle ce qui résulte en une diarrhée aqueuse causée par des enterotoxines thermolabiles (LT) et/ou thermostables (ST).
- EAEC (en vert) s'attachent à l'épithélium de l'intestin grêle et du côlon sous-forme de biofilm épais. Ces bactéries adhèrent entre elles et à la cellule hôte pour former un «pattern» d'adhérence aggrégatif "stacked brick".

- DAEC (en bleu) induisent un effet de transduction de signal sur les entérocytes de l'intestin grêle. Cet effet entraine l'augmentation de projections longues sous forme de doigt, qui s'enroulent autour de la bactérie.
- AIEC (en violet) colonisent la muqueuse intestinale des patients avec la maladie de Crohn's et sont capables d'envahir les cellules épithéliales et de se répliquer dans les macrophages. Les AIEC utilisent le pili type I pour adhérer aux cellules intestinales et le fimbriae polaire long (LPF) pour l'invasion.
- EIEC (en rouge) ciblent les cellules épithéliales du côlon, lysent les phagosomes et progressent à travers la cellule par nucléation des microfilaments d'actine. Après attachement et invasion, les EIEC doivent se diriger latéralement à travers l'épithélium et rentent du côté baso-latéral de la membrane plasmique.

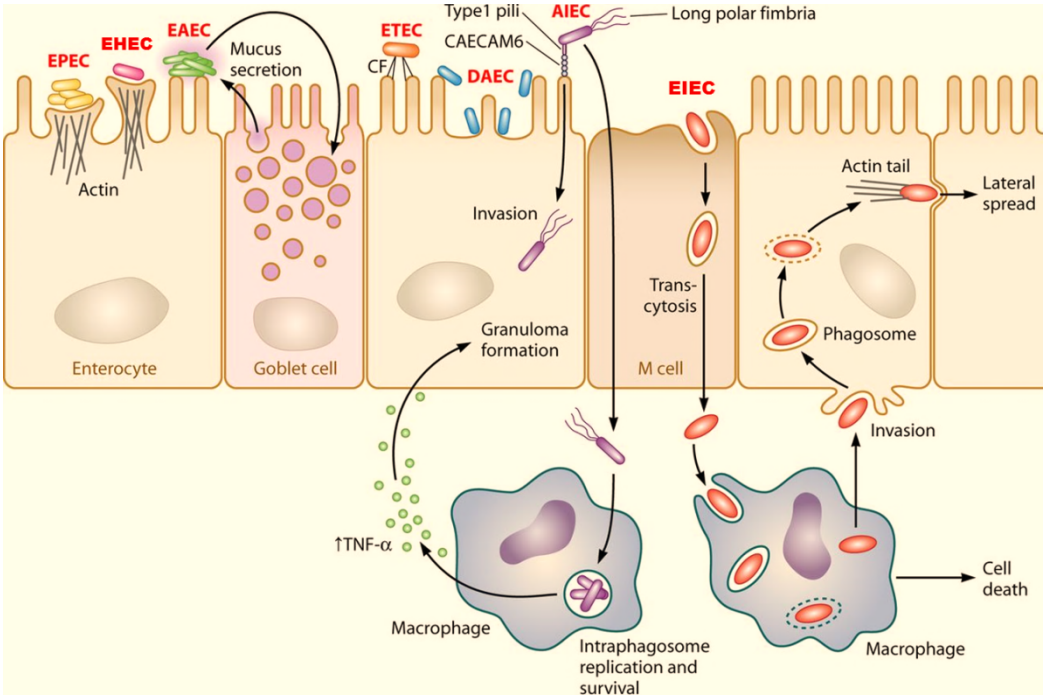

Figure 1. Schéma des mécanismes d'adhérence et colonisation intestinale des sept pathovars d'*E. coli* diarrhéogènes (selon Croxen *et al.,* 2013) [10].

Initialement, les EHEC ont été identifiés comme pathovar distinct d'*E. coli* pathogène à partir de 2 foyers principaux : (i) Le premier a été rapporté par Riley et ses collègues lorsqu'ils ont isolé *E. coli* O157:H7 suite à une épidémie d'origine alimentaire en 1982 associée à une diarrhée aqueuse et sanglante et une colite hémorragique (CH), résultant de la colonisation de la muqueuse intestinale et la libération de toxines [11] (ii) Le second foyer a été signalé en 1983 par Karmali, qui a observé des cas sporadiques de SHU avec des

cytotoxines fécales et des *E. coli* productrices de cytotoxines dans les selles [12]. Le SHU est caractérisé par la triade de l'insuffisance rénale aigüe, la thrombopénie et l'anémie hémolytique micro-angiopathique, et il est généralement précédé par une diarrhée sanglante. Les souches EHEC sont caractérisées par de nombreux facteurs de virulence spécifiques, les deux majeurs étant les Shiga toxines et le locus d'effacement des entérocytes (LEE). Néanmoins, malgré que ce dernier est absent chez certaines souches STEC non-LEE, ces souches sont aussi capables de causer la maladie.

En Amérique du Nord, la répartition géographique de l'infection à EHEC est plus fréquente aux USA dans les états du nord que les états du sud et plus commun dans l'ouest que l'est du Canada [5]. Le sérotype O157:H7 est plus souvent impliqués dans les épidémies et les cas d'infection sporadiques [11,13,14]. En dix ans, entre 1992 et 2002, sur 1645 foyers d'infections intestinales d'origine alimentaire, 44 ont été causées par *E. coli* O157.

1.2. Le sérotype O157:H7

Plus de 100 sérotypes différents d'EHEC ont été incriminés dans les infections chez les humains tels que O26:H11, O86:H34, O111: H8, O113:H21, O117:H14 et O157:H7 [15]. Le sérotype O157 :H7 est reconnu à travers le monde comme le plus infectieux chez les humains et aussi le plus utilisé pour étudier la virulence et pathogenèse des EHEC. Parmi les épidémies et les cas sporadiques d'EHEC, le sérotype O157:H7 est le plus souvent isolé au Canada et É-U et compte pour plus de 90% des cas cliniques [16,17]. Cependant, il est devenu évident que les souches non-O157, notamment les sérogroupes “Big 6” (O26, O45, O103, O111, O121 et O145) sont aussi dangereuses et causent de manière signifiante des infections chez les humains [18].

Une façon d'identifier *E. coli* O157:H7 dans les procédures microbiologiques est l'incapacité de la plupart des isolats de fermenter le sorbitol [19]. Cependant, plusieurs souches fermentant le sorbitol, notamment les STEC O157:H^-, ont également été isolées à partir de patients atteints de SHU en Allemagne [20]. Le taux de récupération d'*E. coli* O157:H7 sur gélose SMAC peut être améliorée par l'enrichissement préalable dans un bouillon sélectif. Les bouillons GN (Hajna) et le milieu soja trypticase complétés par la céfixime (50 ng/ml) et la vancomycine (40 mg/ml) ont été utilisés avec succès [21,22]. En raison de la résistance à la

céfixime et tellurite, la gélose SMAC contenant céfixime et tellurite (CT-SMAC agar) est aussi couramment utilisé pour isoler *E. coli* O157:H7 [23].

1.3. Les effets cliniques de *E. coli* O157:H7

Les EHEC peuvent causer des symptômes cliniques allant d'une infection asymptomatique ou diarrhée légère non sanglante à une diarrhée sanglante ou colite hémorragique (CH), à un SHU, à une purpura thrombocytopénique (PTT) et la mort [24]. Les symptômes de l'infection apparaissent séquentiellement dans les 15 jours suivant l'ingestion de l'aliment contaminé. Les premiers symptômes apparaissent sous forme de diarrhées aqueuses modérées entre 1 et 9 jours après l'ingestion [25]. Dans la plupart des cas, les diarrhées deviennent sanglantes et s'accompagnent d'une déshydratation et de sévères douleurs abdominales. Cette colite hémorragique persiste entre 2 et 10 jours, peut s'accompagner de vomissements mais n'induit généralement pas d'apparition de fièvre. Des complications apparaissent dans 10% des cas une semaine environ après le début des symptômes. Ces complications peuvent mettre en jeu le pronostic vital de l'individu. Elles sont dues à la production de Stx dans l'intestin et se traduisent par des micro-angiopathies thrombotiques de deux types : le SHU et le PTT.

Le SHU se caractérise par une anémie micro-angiopathique hémolytique, une thrombopénie ainsi qu'une insuffisance rénale aiguë. Il a également été observé des troubles du système nerveux central tels que léthargie, convulsions, et encéphalopathie [26]. Le SHU touche essentiellement les enfants de moins de 5 ans et est la première cause d'insuffisance rénale aiguë. Le PTT présente les mêmes caractéristiques physiopathologiques que le SHU mais il diffère du SHU par le fait que les patients présentent une fièvre ainsi que des symptômes neurologiques plus marqués. Cette forme touche plus particulièrement les personnes âgées.

L'impact clinique principal des EHEC provient de leur production des Stx. Celles-ci sont initialement produites par la bactérie dans l'intestin avant de pénétrer dans la circulation générale. L'épithélium intestinal pourrait être endommagé par les Stx, et par le lipopolysaccharide (LPS) bactérien, mais aussi par d'autres médiateurs inflammatoires, ce qui aiderait à la translocation des toxines dans le sang [27]. Cette théorie est renforcée par l'observation des patients atteints de diarrhée sanglante qui sont plus susceptibles de

développer un SHU que ceux avec une diarrhée non-sanglante [24]. Une fois dans la circulation générale, les Stx peuvent être véhiculées par les granulocytes [28], puis se lient aux récepteurs globotriaosylceramide (Gb3) exprimés par les cellules endothéliales qui tapissent les vaisseaux sanguins dans les glomérules rénaux et autres capillaires dans le corps. L'activité des Stx entraine la mort des cellules endothéliales. En essayant de réparer ces dégâts, les fibres plaquettaires peuvent former un caillot qui bloque les capillaires conduisant à un broyage des hématies; ce qui, avec le dépôt de complexes immuns, bloquent les capillaires entrainant la restriction de transfert de l'oxygène vers le tissu local et la mort de ce tissu. Ceci explique le SHU et l'insuffisance rénale, par contre la CH est due à la colonisation de la muqueuse intestinale par les EHEC et la libération subséquente des toxines dans le tractus intestinal.

Chez les enfants, la prévalence des infections par les EHEC O157:H7 d'origine alimentaire est d'environ 3 /100,000 [29]. Cependant, cette faible proportion constitue une menace permanente en santé publique avec un potentiel d'épidémies à grande échelle nécessitant d'importants moyens de surveillance et de prévention. Les risque et l'importance du danger sont justifiés par les coûts élevés des traitements et de prise en charge médicale lors des complications graves et séquelles engendrées telles que les lésions cérébrales, la myocardite aiguë et la déficience rénale chronique nécessitant l'hémodialyse, la transfusion sanguine et parfois la transplantation rénale.

1.4. Traitements et alternatives thérapeutiques expérimentales

Habituellement, après environ une semaine les infections par les EHEC peuvent se résoudre par elles-mêmes [30]. Cependant, actuellement il n'y a pas de moyen pour prévenir le développement de SHU. Les traitements contre les infections à EHEC sont essentiellement symptomatiques tels que la diurèse, hémodialyse et le transfert de plasma frais [31]. Ces traitements diminuent la morbidité chez les patients atteints du SHU ou de PTT [32]. Plusieurs moyens de gestion clinique des infections par les EHEC sont proposés, notamment l'usage de transfusions intraveineuses et le monitoring des plaquettes sanguines et des fonctions rénales [33]. En revanche, l'usage de médicaments d'anti-motilité de l'intestin et d'antidouleur sont proscris. Cependant, l'usage d'antibiotiques lors d'infection à EHEC est encore discuté. En effet, plusieurs études ont montré que l'antibiothérapie est inefficace contre les infections à

EHEC, elle serait même un facteur de risque pour l'apparition du SHU [33]. Les gènes codant les Shiga toxines sont portés par des phages de type λ en phase lysogénique. Certains antibiotiques induisent des cassures de l'ADN bactérien, induisant le cycle lytique du phage, et favorisant l'expression et la synthèse de Stx. Certains antibiotiques bactériostatiques sont à l'étude tels que l'imipenem et le fosfomycine, qui diminuent l'expression et libération des Stx par les EHEC de sérotype O157:H7 et leur cytotoxicité sur les cellules Vero [34,35].

En l'absence de traitement approuvé de SHU, les scientifiques et cliniciens ont mis au point des méthodes alternatives qui peuvent fournir un traitement utile dans l'avenir. Ainsi, des anticorps monoclonaux (AcM) réduisant la cytotoxicité des Stx *in vitro* ont été développés [36]. Le Urtoxazumab est un AcM humanisé (Ac chimérique) dirigé contre Stx2 et qui récemment a été montré pour sa tolérance chez des volontaires en phase 1 d'essai clinique [37]. Alternativement, certains peptides courts peuvent bloquer le transport de Stx2 par les cellules épithéliales [38]. Ces peptides ont un effet protecteur, et diminuent les lésions rénales chez le modèle babouin injecté de Stx2 [39]. La cytotoxicité des Stx1 et Stx2 a été neutralisée avec succès par liaison de ces toxines à un globotriose conjugué au polysaccharide chitosane [40]. Des bactéries recombinantes produisant des analogues structuraux du récepteur de la toxine ont été administrées à un modèle murin. L'administration de ces bactéries supprime toute létalité due à O157:H7 [41]. Les toxines Stx sont de type A_1B_5 et leur action cytolytique nécessite le clivage de la sous-unité A en 2 peptides séparés par une séquence RXXR [42]. Il a été suggèré que ce cleavage est assuré par l'enzyme Furine; membre de la famille des proprotéines convertase (PC) [43,44]. Recemment, le développement et usage d'inhibiteur spécifique à cette PC a empêché avec succès l'effet toxique des Stx sur les cellules Vero [45].

Des stratégies vaccinales contre les EHEC sont aussi en cours de développement [46]. Certaines de ces stratégies visent à réduire le portage et l'excrétion par les hôtes-réservoirs tels que les bovins [47]. Dans ce sens, de nombreuses études ont porté sur des fusions de protéines de différents facteurs de virulence. Parmi les exemples récents il y a : la fusion de Stx2A inactivée avec Stx1B, la fusion Stx2B-Tir-Stx1B-zot (zot; toxine zonula occludens utilisée comme adjuvant mucosal), la fusion Stx2B-Stx1B-EAE et la fusion EspA-EAE-Stx2. Toutes ces fusions ont montré chez la souris soit une protection contre les challenges par EHEC ou Stx ou bien une réduction de l'excrétion de EHEC [48-51]. De plus, pour délivrer d'éventuels vaccins, des mécanismes ont également été explorés, comme la fusion EspA-intimine Tir qui

est exprimée dans les feuilles de tabac et dans les graines de canola, et Stx2B exprimée dans souche vaccinale de *Mycobacterium bovis* BCG [50,52-54].

Globalement, ces stratégies thérapeutiques et vaccinales sont encourageantes. De nombreuses études sont encore nécessaires afin de prouver leur efficacité et réduire leur coût pour lutter contre les infections à EHEC.

1.5. Épidémiologie des EHEC O157:H7

1.5.1. Les sources d'infection

Les ruminants, notamment les bovins, sont considérés comme la principale source d'infection humaine par les EHEC O157:H7 dont ils sont porteurs asymptomatiques (Fig. 2) [55-57]. Chez les bovins, EHEC O157:H7 colonisent la partie terminale du rectum sans provoquer de symptômes évidents [58]. Les STEC sont excrétés par les bovins et leur concentration dans les fèces varie d'un animal à l'autre. Une étude a montré que la concentration en STEC peut varier de 10^2 à 10^5 CFU/g de fèces [59]. Dans les troupeaux, certains bovins excrètent beaucoup plus que d'autres : ce sont les super excréteurs. En effet, ces super excréteurs sont porteurs de STEC à raison de plus de 10^3 CFU/g de fèces et sont responsables de la diffusion des STEC au sein d'une ferme [60]. La prévalence des EHEC O157: H7 dans les fèces d'animaux de troupeaux et d'animaux d'étable est respectivement de 23% et 7,9%, indiquant un risque environnemental considérable pour l'humain et particulièrement via les animaux super excréteurs [61].

Les *E. coli* de sérotype O157:H7 font donc partie des EHEC et des STEC. La récente épidémie de STEC qui a eu lieu en 2011 en Allemagne a été causée par un autre sérotype, l'O104:H4. Celui-ci a été responsable de plus de 823 cas de SHU et 42 décès parmi les 3688 cas d'infection [7]. D'après sa séquence, cette souche fait partie du pathovar EAEC mais portant les phages Stx des EHEC, ce qui expliquait les cas de CH et SHU. En effet, le nombre élevé de cas de SHU peut s'expliquer par une augmentation de libération de Stx dues à une meilleure adhésion ou invasion par cette souche hybride EAEC/STEC. La source présumée de cette épidémie fût les germes de sojas produits localement [62].

1.5.2. La transmission

Les EHEC peuvent être transmises par (i) les aliments et l'eau, (ii) le contact animal-humain, et (iii) le contact de personne à personne (Fig. 2). La transmission d'origine alimentaire est probablement responsable de la plupart des infections.

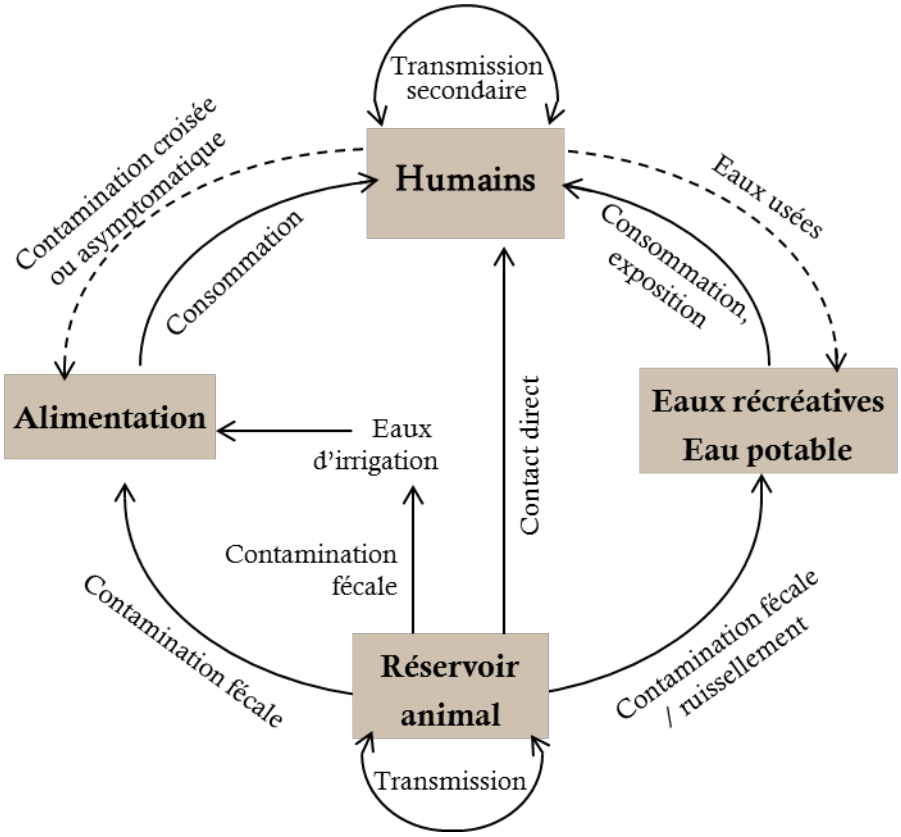

Figure 2. Vue générale des réservoirs potentiels et des modes de transmission des EHEC (selon Croxen *et al.,* 2013) [10].

Les EHEC peuvent être retrouvées dans divers réservoirs animaux et peuvent se propager entre ces derniers et d'autres animaux. Les matières fécales peuvent contaminer les aliments et les eaux d'irrigation, récréatives et/ou potable. L'humain peut être exposé suite à l'ingestion d'aliments ou d'eau contaminée ou par contact direct avec les animaux porteurs. La transmission secondaire peut se produire entre humains, généralement dans les centres de soins ou dans les maisons de retraite. Les aliments peuvent être contaminés par une mauvaise pratique culinaire, par exemple, quand la viande crue pourrait entrer en contact avec d'autres aliments ou bien lorsque l'hygiène des mains des manipulateurs d'aliments est insuffisante. La contamination des eaux potable et récréatives peut se produire via l'exposition aux eaux usées humaines.

(i) L'ingestion d'aliments contaminés crus ou peu cuits, en particulier d'origine bovine comme le bœuf haché (hamburgers) et le lait non pasteurisé, est la voie la plus commune de transmission des EHEC O157: H7. La contamination se produit directement par des matières

fécales ou indirectement, lors du rinçage ou durant les procédures de préparation. L'origine de l'épidémie de 2006 aux USA qui avait causé l'atteinte de 200 personnes, dont 31 avaient développé le SHU, a incriminé la contamination d'épinards emballés.

(ii) Des infections avec les EHEC par contact direct avec l'animal lors de visites de fermes ont été rapportées [63,64]. Même un bref contact physique humain-animal peut transmettre les EHEC, et le lavage des mains est l'unique consigne importante de prévention pour réduire la transmission due au contact humain-animal [65]. Les enfants sont les plus à risque, comme le souligne le nombre de cas de SHU parmi les visiteurs de zoo ou fermes d'exposition [66,67].

(iii) La propagation de la bactérie de personne à personne est possible, et se produit facilement dans des contextes de manque d'hygiène ou au sein des familles où les enfants ont la diarrhée. Dans ces cas, la transmission est probable avec une dose infectieuse d'*E. coli* O157:H7, extrêmement faible.

2. Les facteurs de virulence majeurs chez les EHEC

2.1. Les lésions attachant-effaçant (A/E)

En 1987, Knutton et *al.,* ont démontré l'accumulation de matière dense aux électrons en dessous de bactéries fixées lorsqu'ils ont réalisé des tests d'infection sur une biopsie intestinale humaine [68] (Fig. 3). Knutton a également rapporté que la phalloïdine fluorescente, qui se lie à l'actine, pouvait être utilisée pour visualiser cette matière dense aux électrons en dessous des bactéries EPEC et EHEC attachées à la surface de la muqueuse et que cette coloration correspondait à la taille et la position de chaque bactérie adhérente [69]. Par conséquent, cette coloration spécifique à la phalloïdine pouvait être appliquée pour cribler, par mutagenèse aléatoire, les gènes responsables du phénotype de lésions A/E.

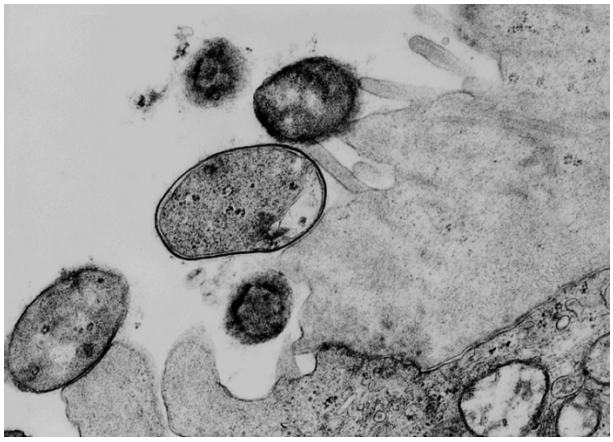

Figure 3. Lésions A/E des cellules épithéliales intestinales du lapin causées par *E. coli* (selon Goosney *et al,.* 1999) [70].

Les EHEC colonisent la muqueuse intestinale en s'attachant à l'épithélium intestinal de l'hôte et provoquent des lésions A/E. Ces dernières sont caractérisées par l'effacement de la bordure en brosse et la polymérisation de l'actine en dessous des bactéries [71].

La bactérie adhérente s'attache étroitement sur un piédestal formé par polymérisation de l'actine de l'hôte [68]. La formation des lésions A/E dépend du système de sécrétion type 3 (SST3) [72], l'intimine: protéine adhésive de la membrane externe [73] et de son récepteur Tir, qui est transloqué dans la cellule hôte [74]. Tous ces éléments sont codés dans l'îlot de pathogénicité OI#148 appelé le locus d'effacement des entérocytes (LEE) [75].

2.2. Le LEE et le système de sécrétion type 3 (SST3)

2.2.1. Le LEE

En plus de la structure génomique de base commune entre les souches commensales et pathogènes, les souches pathogènes ont acquis d'autres gènes encodés par les îlots de pathogénicité (PAI) qui rendent la bactérie plus virulente et permettent son adaptation à de nouvelles niches environnementales. Le LEE code pour un SST3, divers translocateurs et effecteurs, l'intimine et son récepteur Tir [73,76]. Les EHEC et EPEC avec de nombreux autres pathogènes à Gram négatif, utilisent un SST3 pour sécréter et injecter des protéines effectrices dans le cytosol des cellules hôtes. Ce processus entraine la formation de lésions A/E [77].

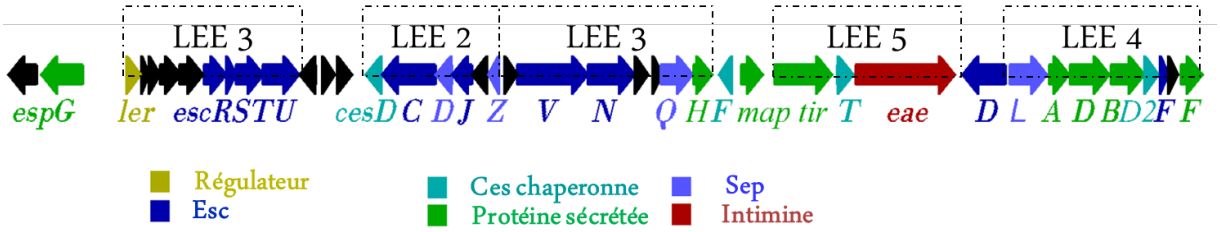

Figure 4. Schéma du LEE chez les EHEC

Le LEE, schématisé dans la Figure 4 est d'environ 43 kb et est composé de 54 ORFs qui forment cinq principaux opérons (LEE1-5) [78]. Les LEE 1 à 3 codent pour les composants de l'appareil de base du SST3 (EscC, J, R, S, T, U, V) [8,79,80] (Fig. 5). Le LEE4 code pour EspA, B, D, F [81-88], SepL [89] et EscF [90]. Le LEE5 code une adhésine de la membrane externe appelée intimine, son récepteur transloqué Tir, et une chaperonne de Tir appelé CesT [91]. Tir est situé à la membrane plasmique de la cellule hôte et forme une structure en épingle à cheveux qui se lie à l'intimine [74]. Après l'assemblage de l'appareil basal du SST3 et du complexe «d'aiguille» à EspA, EspB et Tir, ceux-ci sont transloqués dans la cellule épithéliale hôte où Tir sera intégré dans la membrane plasmique. La liaison de Tir à l'intimine sur la surface de la bactérie est responsable de la réorganisation du cytosquelette de la cellule hôte qui entraine la formation des lésions A/E pendant l'infection [92,93]. SepL est requise pour la formation de l'appareil de translocation intact et la sécrétion des protéines Esp [89]. SepD est la seule protéine qui a été démontrée interagir avec SepL par le système

double-hybride chez la levure et est considérée comme ayant une fonction associée à SepL [94].

2.2.2. Le SST3

Les bactéries pathogènes à Gram négatif: *Yersinia* spp, *Salmonella* spp, *Shigella* spp, et les EPEC et EHEC peuvent toutes exprimer des SST3s spécifiques mais connexes. Ces bactéries utilisent ce système pour transférer des protéines et des effecteurs dans la cellule hôte. *Yersinia* et *Shigella* ont une «aiguille» courte pour injecter les effecteurs depuis l'intérieur de leurs cellules hôtes. Cependant, les EPEC et EHEC utilisent un SST3 différent dans lequel une structure filamenteuse s'étend à partir de l'«aiguille». La nécessité de cette structure supplémentaire n'est pas évidente mais peut agir en tant que première adhésine [95] pour permettre ensuite au SST3 de pénétrer le mucus ou traverser les microvillosités. Le modèle du SST3 des EHEC est illustré à la figure 5.

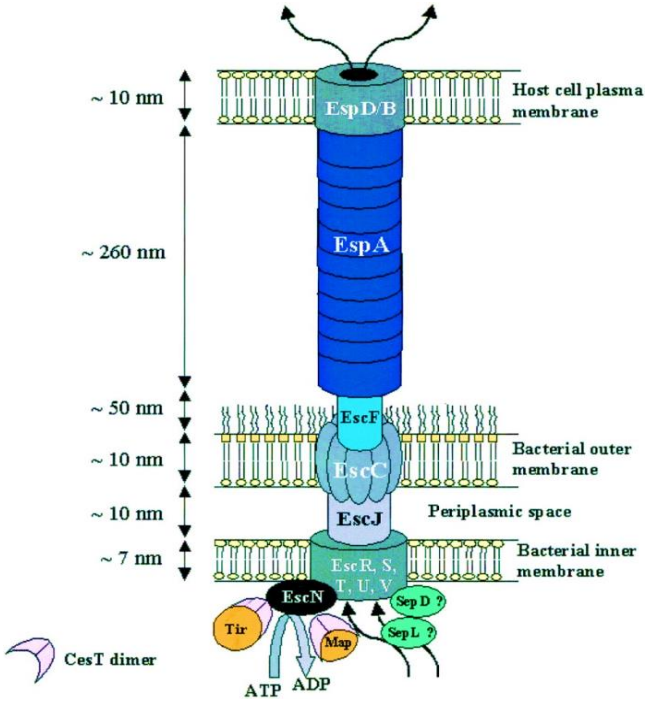

Figure 5. Schéma du système de sécrétion de type 3 des EHEC (selon Garmendia *et al.,* 2005) [8].

La partie basale du SST3 est composée de la sécrétine EscC, des protéines de membrane interne EscR, EscC, EscT, EscU et EscV ainsi que de la lipoprotéine EscJ qui connecte les structures en anneaux des membranes internes et externes. EscF constitue la structure de la seringue tandis que les sous-unités EspA polymérisent pour former le filament EspA. EspB et EspD forment le pore de translocation dans la membrane de la cellule hôte connectant ainsi la bactérie avec la cellule

eucaryote via le filament EspA. L'ATPase cytoplasmique EscN fournit l'énergie au système en hydrolysant les molécules d'ATP en ADP. SepD et SepL sont des composants cytoplasmiques du SST3. CesT est une protéine chaperonne nécessaire à la sécrétion de Tir en favorisant le contact physique avec EscN.

2.2.2.1. Translocon du SST3

Une fois la structure en «aiguille» en position, l'assemblage du translocon commence. Ce dernier est codé par l'opéron LEE 4. EspA est la principale composante de l'extension filamenteuse. EspB and EspD forment un pore (ou trou de passage) à l'extrémité du filament à EspA. EspB et EspD ont des tailles similaires et sont les principales protéines sécrétées par SST3 qui peuvent être détectées dans le surnageant des EHEC. EspD influence la longueur du filament EspA, favorise l'adhésion aux premiers stades de l'infection; et génère le pore de translocation dans les cellules hôtes une fois le contact établi [82]. Par ailleurs, la suppression de SepL et SepD provoque l'échec de l'export du translocon EspA indiquant leur nécessité pour la translocation [96,97].

2.2.2.2. Le translocon sécrété et les effecteurs codés par le LEE

Le LEE4 code pour des protéines effectrices EspA, B, D, F, SepL et EscF. L'expression du filament EspA est hétérogène chez les souches EHEC O157, isolées de bovins et de patients humains [92]. La proportion de la population bactérienne exprimant les filaments EspA est proportionnelle au niveau de sécrétion d'EspD. À l'échelle de la bactérie, l'expression du LEE4 est régulée par un mécanisme post-transcriptionnel, qui contrôle la translation de l'ARNm *espADB*.

D'autres effecteurs codés par le LEE sont impliqués dans la modulation du cytosquelette de l'hôte, notamment la Map, SepZ, EspH, EspF, EspO et EspZ.

L'effecteur Map est retrouvé au niveau mitochondrial et joue un rôle important au stade initial de l'infection en déclenchant des déformations et dommages des mitochondries [98]. Indépendamment du ciblage mitochondrial, Map est aussi responsable d'altérations des jonctions serrées et de la fonction de la barrière intestinale [99]. Deux autres membres de la famille Map ont été découvertes, et ont été désignées EspM1 et EspM2 [100]. EspH se trouve sur la membrane de la cellule hôte dont il affecte les structures cytosquelettiques d'actine, comme les filopodes et la formation en piédestal [101]. SepZ est décrite comme protéine

effectrice codée par le LEE. Toutefois, aucune étude n'a montré si sa translocation serait associée à une fonction spécifique. EspF est une protéine effectrice riche en proline pouvant perturber la barrière intestinale, augmenter la perméabilité membranaire et induire la libération du cytochrome C [102-105]. EspO renforce l'adhésion des cellules hôtes à la membrane basale en interagissant avec la kinase liée aux intégrines [106]. En bloquant la phosphorylation de Paxillin et la kinase des adhésions focales (FAK), EspO peut inhiber le «turnover» ou recyclage des adhésions focales lors de la motilité cellulaire. EspZ a une fonction similaire à celle de EspO dans la prévention de détachement de la cellule hôte par interaction avec la protéine de l'hôte CD98 et l'amélioration de la phosphorylation des kinases FAK et Akt, ce qui renforce la stabilité des adhésines focales lors de l'infection [107]. Récemment EspZ a été décrit comme inhibiteur de la formation d'actine en piédestal en bloquant la translocation de Tir, Map et EspF [108], suggérant son rôle dans l'équilibre de la concentration intracellulaire des effecteurs afin de prévoir la toxicité chez l'hôte durant le cycle infectieux.

2.2.2.3. Les effecteurs non-codés par le LEE et secrétés par le SST3

En plus des effecteurs codés localement par les opérons LEE, le génome des EHEC O157 encode de nombreux autres effecteurs non-codés par le LEE appelés Non-LEE (Nle).

Dans la famille NleG, les protéines NleG ont beaucoup d'homologues sécrétées par le SST3 des souches EHEC O157:H7. Ces effecteurs font partie d'une famille d'ubiquitine ligase U-Box E3 [109]. Les effecteurs NleG sont probablement liés au «turnover» des protéines de la cellule hôte et/ou d'autres effecteurs de type III par le protéasome via la voie d'ubiquitination de la cellule hôte [109].

NleA-F sont aussi sécrétées par SST3, NleA est transloquée dans les cellules hôtes, localisée dans l'appareil de Golgi et est requise pour la virulence des EHEC [110]. En revanche, NleB et NleE empêchent l'activation de NF-κB en inhibant la dégradation de IκB et la translocation nucléaire de p65 [111-113]. NF-κB régule l'expression des gènes de cytokines. Par ailleurs, NleC peut bloquer la fonction de NF-κB ce qui augmente la sécrétion d'effecteurs [114].

En plus des effecteurs décrits ci-dessus, EspJ est un effecteur encodé par le phage CP-933U et sa sécrétion par *E. coli* influence le SST3 et la virulence des EHEC [115]. Bien que la

protéine EspJ ne soit pas essentielle pour une activité de lésion A/E, elle a un effet sur la clairance du pathogène depuis le tractus intestinal de l'hôte. Cela laisse supposer son rôle dans la survie de l'agent pathogène dans l'hôte et sa transmission [115]. D'autres effecteurs ont été décrits notamment NleD, EspW et EspV. L'expression de la protéine EspV dans les cellules de mammifères cause des altérations morphologiques drastiques liées à une condensation nucléaire et formation de projections dendritiques [116].

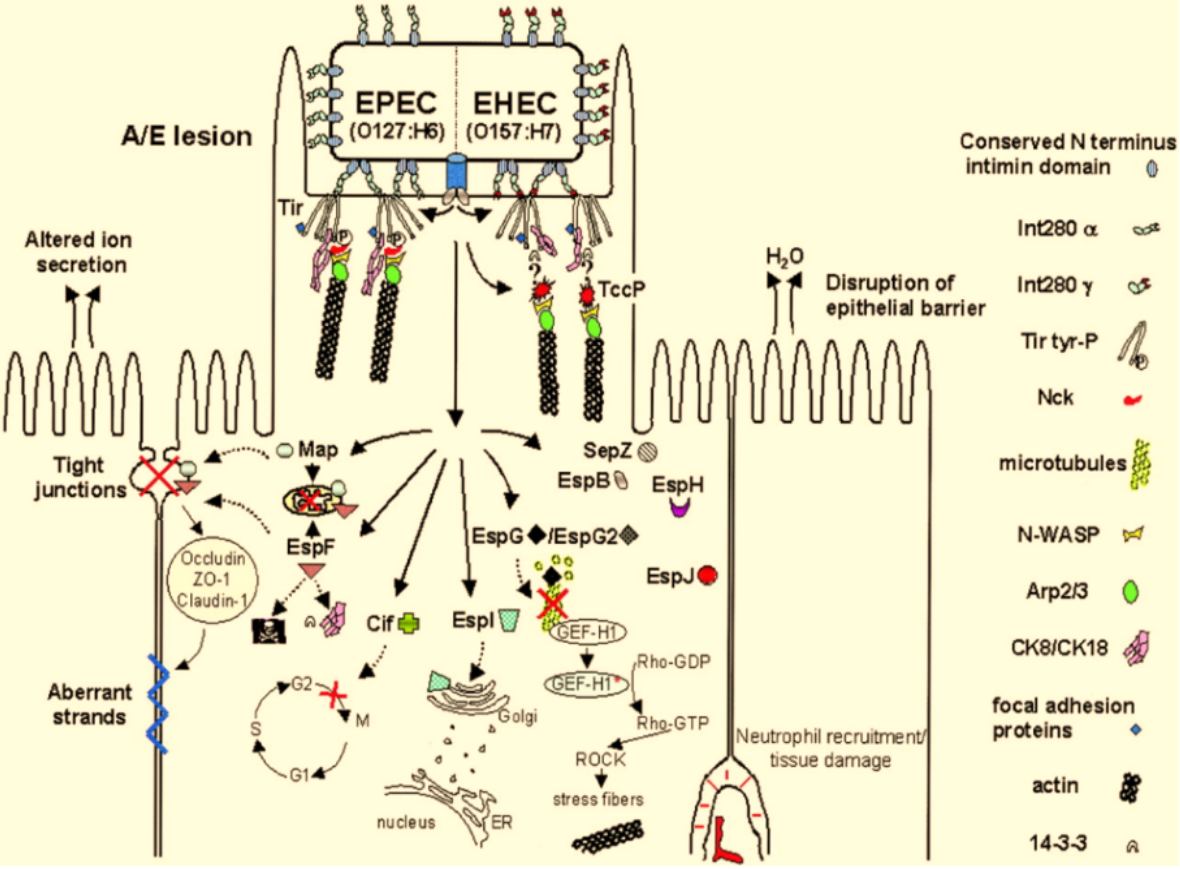

Figure 6. Translocation des effecteurs des EHEC et EPEC dans le cytosol de la cellule hôte (selon Garmendia et al. 2005) [8].

Ces effecteurs déclenchent des réarrangements du cytosquelette (Tir, EspH, EspF et EspG), une perturbation de la barrière épithéliale (EspF et Map), la cytotoxicité (EspF et Cif), et une réponse de la cellule hôte qui ultimement génère une diarrhée aqueuse. Différemment des EPEC, le Tir des EHEC est non-phosphorylé au résidu tyrosine et n'a pas besoin d'interagir avec Nck pour déclencher la polymérisation de l'actine au site d'adhésion. En revanche les EHEC transloquent l'effecteur TccP/EspFu, qui remplace l'activité Nck essentielle à la réorganisation de l'actine du cytosquelette sous-jacent aux bactéries adhérentes.

2.2.2.4. La chronologie de sécrétion

Après l'assemblage du translocon, la sécrétion des effecteurs commence. Il a été rapporté que cette sécrétion obéit à un ordre important et précis résultant de relations synergiques et antagonistes ordonnées entre les effecteurs de type III [117]. En se basant sur l'efficacité de la translocation des effecteurs, cette dernière a été démontrée chez les EPEC suivant l'ordre: Tir > EspZ > EspF > EspH > EspG > Map [118,119]. Il est considéré que ces effecteurs fonctionnent de manière coordonnée au sein de la cellule hôte et leur différence d'efficacité de translocation conduit à une hiérarchie de leur transfert et activité dans la cellule hôte [101,120]. Ce processus ordonné est dicté par la concentration intracellulaire de chaque effecteur et aussi par leur affinité aux chaperonnes cytoplasmiques. En effet, la fonction de plusieurs effecteurs requiert des protéines chaperonnes. Par exemple, CesT est une chaperonne impliquée dans la maximisation de l'efficacité de translocation de Tir dans la cellule hôte et donc de l'effet A/E [121]. Autre protéine chaperonne, SepD qui se lie à SepL, est aussi essentielle pour la sécrétion d'effecteurs et à la formation d'A/E. La délétion du gène *speD* entraine l'échec de la sécrétion.

En résumé, chez les EHEC le SST3 comprend un appareil de base avec ses composants protéiques présents à la fois dans la membrane bactérienne interne et externe, et un complexe d'aiguille connu sous le nom de translocon, qui permet l'injection de protéines effectrices à travers la membrane de la cellule hôte [92].

2.3. La régulation du SST3

2.3.1. Les régulateurs codés par le LEE

L'expression du LEE est régulée de manière complexe en réponse à de nombreux régulateurs et facteurs environnementaux tels que le pH, le glucose, le fer et la température [122]. L'expression du SST3 est un processus à plusieurs étapes contrôlées par des facteurs et des signaux; ceux connu jusqu'à maintenant sont illustrés dans la figure 7.

Le Ler (régulateur global du LEE) est une protéine de 15 kDa codée par le premier gène de LEE1 et régule directement les gènes du LEE et d'autres gènes ailleurs dans le génome. Ler est un «dé-répresseur» qui lève la répression des opérons du LEE par le régulateur non-spécifique H-NS [79]. Le Ler a un rôle crucial dans le contrôle de l'ensemble

SST3 et des gènes impliqués dans les lésions A/E [123]. Sa transcription est régulée à la fois positivement et négativement par deux autres régulateurs exprimés par le LEE; GrlA et GrlR [124]. Ils ont tous deux un effet sur le promoteur du *ler* et régulent l'expression LEE à travers Ler. GrlR agit aussi sur l'expression génique du LEE en interagissant avec GrlA.

En plus de ces régulateurs, la réponse à des facteurs environnementaux, en partie médiée par *pchA, pchB* et *pchC* (homologues de *perC* chez les EPEC), peut également induire une régulation positive du SST3 [125]. À travers PchA, B, C, les protéines GrlA et GrlR sont stimulées, et par conséquent, affectent le niveau d'expression du SST3.

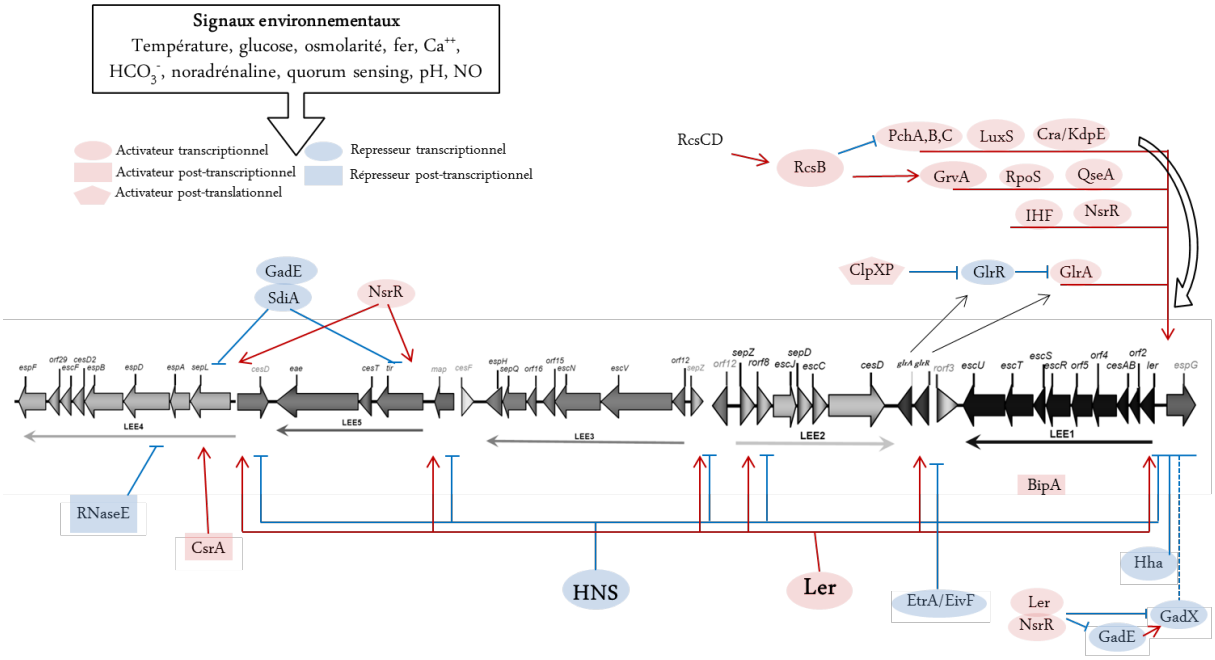

Figure 7. Régulation du LEE chez les EHEC.

Ce schéma montre que le LEE codant le SST3 est soumis à une régulation complexe par plusieurs facteurs et signaux environnementaux (voir détail dans le texte).

2.3.2. Les régulateurs non-codés par le LEE

Chez les EHEC, sept homologues de PerC sont codés dans des îlots-O (OIs); PchA-E, PchX et PchY. Les gènes *pch* forment trois classes différentes en fonction de la similarité de séquence. Le *pchA, pchB* et *pchC* appartiennent à la classe 1 partageant une grande similitude de séquence, ce sont des régulateurs positifs du LEE1 [126]. Il a été montré que PchA-C facilitent l'activation des gènes encodés sur les OIs [127]. PchA est réprimé par le système phospho-

relais RcsBCD. Toutefois, RcsB régule positivement et indirectement la transcription du LEE via l'activation de GrvA, qui est un activateur EHEC-spécifique [128]. De plus, les gènes *pchA,B,C* sont contrôlés et activés par la réponse stringente. Cette régulation, médiée par l'alarmone ppGpp, est responsable de l'augmentation de l'expression du LEE et du SST3 lorsque la bactérie est en phase de culture stationnaire et/ou lors d'épisode de famine environnementale [103]. Des études utilisant le profilage transcriptionnel et l'immunoprécipitation de la chromatine, montrent que PchA et/ou Ler se lient et activent la transcription de nombreux gènes d'effecteurs Nle et des gènes horizontalement acquis codant pour d'autres facteurs de virulence [129]. Les deux autres classes des gènes *pch* sont *pchD* et *pchE*. La fonction de ces 2 gènes n'est pas claire, et il a été démontré que *pchD* et *pchE* ne peuvent pas activer l'expression du LEE1 dans une souche K-12 [127]. Généralement, les gènes de la famille *pch* sont codés sur les régions «truffée» de prophages qui codent également des protéines effectrices Nle. Les gènes *pch* induisent l'expression de ces effecteurs en même temps qu'en s'assurant de la production du SST3, permettant ainsi leur transport. Ces régions encodent aussi des Prs (régulateurs de sécrétion de phage) qui semblent réprimer les principaux effecteurs codés par le LEE pour permettre aux Nles d'être compétitifs pour la translocation [130].

Lors du processus infectieux, les EHEC résistent au milieu gastrique très acide. Chez les EHEC O157:H7, GadE est le régulateur principal de la résistance à l'acidité. GadE inhibe l'adhésion aux cellules épithéliales intestinales Caco-2 en réprimant la synthèse des protéines codées par le LEE [130,131]. L'effet répresseur de GadE sur LEE et indirect via l'activation de l'expression de *gadX*, et GadX réprime l'expression de *ler*. De plus, GadE réprime aussi l'expression des opérons LEE4 et LEE5 indépendamment de Ler. En retour, Ler réprime l'expression de *gadE* et de *gadX* [132].

Le monoxyde d'azote (NO) est un effecteur majeur de la réponse immune innée, produit en particulier par les cellules épithéliales intestinales. Il avait été montré que le NO réprime l'expression du LEE et active celle de *gadE* et de *gadX* [132]. La présence de NO inactive le régulateur NsrR, qui est un activateur direct et indirect du LEE. NsrR active directement l'expression des opérons LEE1, LEE4 et LEE5 et réprime indirectement l'expression de *gadE* et *gadX* [132]. Ainsi, le NO réprime directement l'expression du LEE en

supprimant la fixation de NsrR aux promoteurs du LEE1, LEE4 et LEE5, et indirectement en activant l'expression de *gadE* et donc de *gadX*.

Le SST3 est également régulé par les hormones de l'hôte, adrénaline et la noradrénaline (Ad/NA), via le régulateur du «quorum-sensing» QseA, qui contrôle le niveau d'expression de *ler* et *grlA*. De plus, QseA à son tour est régulé par les signaux de Ad/Na et l'auto-inducteur-3 (AI-3) à travers le système à deux composants QseC-B [80].

2.4. Les Shiga toxines (Stx) et les phages encodant ces toxines

2.4.1. Les Stx

Les EHEC, étant des STEC, sont responsables de CH et SHU associés à l'activité des Stx [133]. Il existe deux groupes immunologiquement non-réactifs de toxines Stx, Stx1 et Stx2 [134]. Une souche EHEC peut produire Stx1, Stx2 ou les deux, et parfois même de multiples formes de Stx2. Chez les EHEC, Stx1 présente une homologie de séquence avec Stx de *Shigella dysenteriae* type 1. Stx1 de différentes origines a plus de 99% d'homologie de séquences nucléotidiques et malgré cette grande homogénéité, plusieurs variants *stx1* ont été décrits: *stx1, stx1c, stx1d* [135-137].

La toxine Stx2 est la plus répandue parmi les STEC. Des études réalisées *in vitro* sur des cellules endothéliales microvasculaires rénales [138], et *in vivo* sur des modèles animaux [139,140], indiquent que Stx2 est une toxine plus puissante que Stx1. Ces résultats corroborent les études épidémiologiques indiquant que les souches responsables des cas les plus sévères chez l'Homme, comme les souches de sérotype de O157:H7, possèdent majoritairement la toxine Stx2 [141,142].

Stx2 est plus variable et subdivisée en sous-classes dont la nomenclature n'est pas très claire, et qui porte souvent à confusion, les auteurs ne donnant pas toujours le même nom au même variant : Stx2, Stx2c, Stx2d, Stx2-vha (Stx2d1), Stx2-vhb (Stx2d2), Stx2-vhc, Stx2e, Stx2f, Stx2-OX3A, Stx2-OX3b, Stx2-O111, Stx2-O48, Stx2-O118, Stx2-NV206 [143-147]. Tous ces variants décrits sur la base de différences de séquence nucléotidique ne varient que très peu en termes de séquence protéique, et par conséquent il est probable que ces différences soient muettes en termes d'activité biologique. Les souches portant les variants Stx2, Stx2c et

Stx2d (Stx2-vha et Stx2-vhb) sont les plus associées aux pathologies les plus sévères comme le SHU, et sont majoritairement portés par les souches de sérotype O157:H7 qui sont les plus virulentes pour l'Homme [148]. Les variants Stx2e ou Stx2f sont retrouvés dans les souches de séropathotypes C, D et E et sont associés à des pathologies telles que les diarrhées ou du portage asymptomatique [141,143,149,150]. Les STEC produisant Stx2e sont pathogènes pour le porc, provoquant la maladie de l'œdème [151]. Les souches produisant Stx2f sont très rarement isolées de l'Homme, leur réservoir naturel étant le pigeon [152].

La structure de base des sous-unités A-B est conservée dans tous les membres de la famille de Stx. Les Stx comprennent une sous-unité A de ~32 kDa et 5 sous-unités B de ~7.5 kDa chacune. La sous-unité A est composée de deux peptides; A_1 a une activité enzymatique et A_2 est responsable de la liaison de la sous-unité A au pentamère de sous-unités B. La sous-unité B reconnait un récepteur glycolipidique spécifique, le globotriaosylceramide (Gb3), à la surface des cellules eucaryotes. Les Gb3 sont la principale cible des Stx, alors que la variante Stx2e utilise Gb4 comme récepteur. Ces récepteurs sont retrouvés à la surface de cellules épithéliales rénales; les néphrocytes, les cellules plaquettaires, les érythrocytes ainsi que sur d'autres types cellulaires. Une fois la toxine liée à la membrane cellulaire, l'holotoxine est internalisée par endocytose puis livrée à l'appareil de Golgi. Par la suite, la toxine est transportée au réticulum endoplasmique (RE) et finalement au cytosol [153]. Le clivage catalytique de la sous-unité A intervient d'abord au niveau de l'endosome et du réseau trans du Golgi par l'intermédiaire d'une proproteine convertase, la furine, puis par un mécanisme secondaire dans le RE par l'intermédiaire de la protease calpaïne [43,44,154]. Dans le cytoplasme, la sous-unité A_1 clive l'ARN ribosomal 28S d'eucaryotes, ce qui inhibe la synthèse protéique [155]. La conséquence de la cessation de synthèse protéique est la mort des cellules qui possèdent les récepteurs Gb3 (ou Gb4 pour Stx2e), telles que les cellules épithéliales intestinales ou rénales, cellules endothéliales, cellules Vero et HeLa. Les toxines Stx1 et Stx2 partagent 57% d'homologie de séquence d'acides aminés en sous-unité A et 60% en sous-unité B [156].

Les données épidémiologiques démontrent que Stx2 a un rôle plus important que Stx1 dans le développement de SHU. Les souches d'*E. coli* O157:H7 portant Stx2 seule, semblent être plus associées au SHU que les souches produisant Stx1 ou Stx1 et Stx2 [157]. Stx2 et non

Stx1, est critique pour les lésions rénales chez la souris et aussi toxique pour les cellules endothéliales rénales en culture [138,158]. Cette différence de toxicité entre Stx1 et Stx2 pourrait être expliquée par la différence de séquence d'acides aminés de leur sous-unité A [159].

2.4.2. Les phages Stx et cycles lytique et lysogénique de phage lambda

Les phages jouent un rôle important dans l'évolution et la virulence de plusieurs pathogènes tels que *E. coli*, *Clostridium botulinum*, *Staphylococcus aureus* et *Pseudomonas aeruginosa* [160]. L'évolution de certaines bactéries est étroitement liée à celle des bactériophages. Beaucoup de ces bactériophages portent des gènes «cargo» appelés aussi gènes «idiots» ou gènes de conversion lysogénique. Ces gènes ne sont pas nécessairement requis pour le cycle de vie du phage, alors pourquoi sont-ils présents sur les bactériophages? En effet, beaucoup de ces gènes «cargo» codent pour des facteurs de virulence et contribuent au changement des phénotypes ou au «fitness» des bactéries lysogènes. Par conséquent, ces souches sont plus compétitives dans l'hôte ou dans d'autres environnements, et peuvent être plus pathogéniques pour l'humain.

Dans le génome de la souche *E. coli* O157:H7 EDL933, Stx1 et Stx2 sont codées par 2 prophages différents: respectivement CP-933V et BP-933W [161,162]. Les séquences de ces phages présentent une grande homologie à la famille de phage lambda (λ) [161]. La taille du génome de ces différents phages est variable et a été estimée entre 47 kb et 70 kb [163,164].

Durant leur évolution, les souches EHEC ont intégré dans leur génome plusieurs phages. Dès son entrée dans la bactérie, le phage prend la décision lyse ou lysogénie en fonction des signaux de l'environnement et du nombre de phages infectant par cellule [165]. Si la voie lysogène est activée, l'ADN du phage s'intègre dans le génome de l'hôte, ce qui s'accompagne par une extinction de la voie lytique. De plus, l'ADN du prophage se réplique avec le chromosome de son hôte lors de la division bactérienne contribuant à l'immunité de cette dernière contre d'autres phages. Le phage à l'état lysogénique est très stable, mais répond à l'induction SOS suite aux dommages d'ADN dans certaines conditions extrêmes. Quand la réponse SOS est induite, le passage de l'état lysogène à lytique se produit suite au clivage de CI par RecA [166]. La cellule hôte sera lysée après la réplication du phage. Dans certains cas,

quelques-unes de ces cellules hôte induites peuvent entrer dans un cycle lytique avorté subissant un «curetage» de l'ADN du phage.

Les phages λ codant les Stx partagent une structure génomique similaire. La région centrale du circuit de régulation est appelée région immunitaire. Cette dernière habituellement localisée entre les gènes N à O du phage λ contient 2 gènes codant des protéines qui lient l'ADN spécifiquement; CI et Cro, et six sites d'opérateurs auxquels CI et Cro se lient [167]. En comparant 8 phages lambdoïdes, dont trois sont des phage producteurs de Stx, une grande similitude a été observée dans leur région immunitaire habituellement conservées [168].

2.5. Régulation des phages Stx et transcription des gènes *stx*

Les phages portant les gènes *stx* sont inductibles comme les autres phages de type lambda. Cette induction passe par un système bactérien de réponse au stress : le système SOS. Ce système est induit dans la cellule bactérienne lorsqu'il y a accumulation d'ADN simple brin dans la cellule suite à des dommages de l'ADN lors d'une irradiation aux UV, lors d'une alkylation de l'ADN, ou lors de tout autre processus d'altération de l'ADN [169]. La réplication de l'ADN est alors bloquée, et la réponse SOS se met en place (Figure 8).

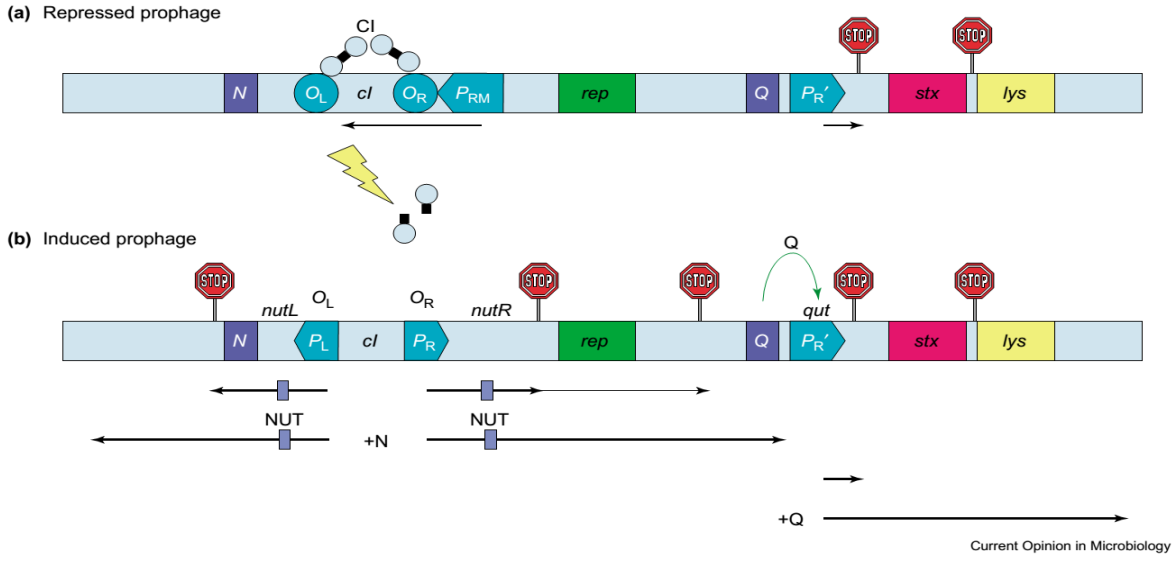

Figure 8. Région de régulation précoce des phages encodant *stx* (selon Waldor et Friedman, 2005) [170]. (a) La répression lysogénique: La transcription qui initie la cascade de régulation (montrée en [b]) est bloquée aux opérateurs O_L et O_R par le répresseur CI. En absence de RecA, CI initie la transcription depuis P_{RM} et synthétise le répresseur pour maintenir le cycle lysogénique. En absence de Q, la transcription initiée au promoteur $P_{R'}$ se termine immédiatement en aval du

terminateur. Le signal ⚡ indique l'action d'agents qui provoquent la réponse SOS conduisant à l'augmentation de production et d'activation de RecA qui facilite l'auto clivage de CI et lève la répression. (b) L'induction lytique: La cascade de régulation commence par l'initiation de la transcription aux promoteurs P_L et P_R qui se termine après la synthèse d'un message court. Cette transcription précoce permet l'expression de N, qui agit aux sites NUT de l'ARN et modifie l'ARNP en une forme qui transcende les terminateurs. Ensuite Q s'exprime et agit sur le site *qut* de l'ADN permettant ainsi de modifier l'initiation de la transcription au $P_{R'}$ en une forme résistante à la terminaison. Ceci permet la transcription des gènes en aval comme les gènes *stx* et *lys* et aussi la plupart des gènes requis pour la production du phage viable.

Le régulateur majeur de la réponse SOS est la protéine RecA, présente en faible quantité mais de façon constante dans la cellule. En présence d'ADN simple brin, la protéine RecA se fixe sur l'ADN endommagé pour former des filaments et acquiert ainsi une activité protéase. RecA ainsi activée induit l'autoclivage du répresseur LexA qui réprime les gènes du régulon SOS. Parmi les gènes du régulon SOS, les gènes *uvr* sont impliqués dans la réparation des dommages liés aux UV, et le gène *sulA* inhibe la division cellulaire afin de laisser le temps nécessaire à la réparation de l'ADN [171]. Ces gènes possèdent sur leur promoteur un site de fixation de LexA nommé boîte SOS. Il a été rapporté que 1% des gènes exprimées chez *E. coli* possèdent une boîte SOS sur leur promoteur [172]. La réponse SOS est donc un phénomène cellulaire global dont l'activation est sous la dépendance de nombreux phénomènes physiques ou biologiques qui peuvent aller d'une simple variation de pH à l'action d'antibiotiques ayant un effet sur l'ADN, comme la mitomycine C ou les quinolones.

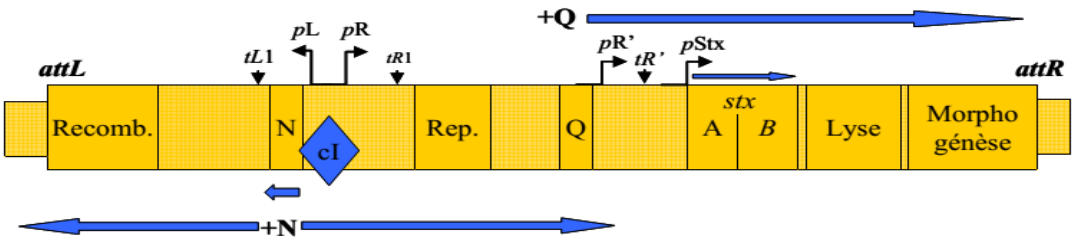

Figure 9. Structure du phage BP933W portant les gènes *stx2* (Plunkett et al. 1999) [173].

p:promoteur, t:terminateur, cI: répresseur, N, Q : antiterminateurs, attL et attR: jonctions prophage ADN bactérien. Les gènes *stx2* sont situés en amont des gènes de la lyse du phage. En phase lysogénique, la réplication du phage est réprimée par CI. Les gènes *stx* sont faiblement exprimés sous l'effet d'un promoteur constitutif. Lorsque le phage est induit, le répresseur CI subit un autoclivage et les deux protéines N et Q produites permettent une forte expression des gènes de la réplication, de la lyse et de la morphogénèse du phage ainsi que l'expression des gènes *stx2*.

Les protéines régulatrices CI et Cro contrôlent le développement lytique ou lysogénique avec un système de commutateur génétique «bistable». Le régulateur CI maintient l'état lysogène en contrôlant l'expression des trois promoteurs P_L, P_R and P_{RM} tandis que Cro régule le développement lytique en baissant le niveau de CII, qui active la transcription *cI*. Un système immunitaire est habituellement utilisé par le phage λ pour prévenir l'infection multiple de lysogènes. La protéine CI est essentielle pour ce système immunitaire. CI garde un certain niveau d'expression qui réprime les gènes d'autres phages impliqués dans leur intégration dans le génome de l'hôte et leur cycle de réplication. En conséquence, l'intégrase des autres phage λ est inhibée donnant ainsi un seul lysogène par bactérie [174].

Les gènes de Stx sont habituellement retrouvés immédiatement en aval du gène Q. Stx est produite à la fin du processus lytique. L'expression de Stx est généralement réprimée dans les bactéries lysogéniques. Cependant, une fois le programme lytique du phage est induit, RecA provoque l'autoclivage du répresseur CI, ceci lève la répression la transcription aux promoteurs précoces P_L et P_R conduisant au final à l'expression de Stx via le jeu de transcription du terminateur/anti-terminateur (Figs. 8 et 9) [170,175]. Les gènes *stx* sont situés en aval d'un autre promoteur tardif du phage, $P_{R'}$ [173,176]. Un terminateur de transcription assure l'inefficacité de ce promoteur lors de la lysogénie. En revanche, lorsque le phage est induit, l'expression des gènes précoces est rendue possible. L'élongation de la transcription commence par l'anti-terminateur N qui se lie au site ARN 'N utilization' (NUT) et active l'initiation de la transcription au promoteur P_R. Ainsi, les terminateurs sont substitués et l'anti-terminateur Q est activé. Quand l'ARN NUT est synthétisé, différentes protéines associées à NUT s'associent ensemble avec l'ARN polymérase (RNAP), formant un complexe qui substitue l'aval du signal de terminaison de la transcription. Quand le promoteur P_R est actif, la protéine anti-terminateur Q est produite. Cette dernière reconnait une séquence d'ADN au site *qut*, partiellement contenue dans $P_{R'}$ [177], ce qui permet à la RNAP de continuer sa lecture et de transcrire les gènes tardifs du phage, dont les gènes *stx* et les gènes de lyse. Par ailleurs, les études génomiques ont identifié un autre promoteur, situé directement en amont des gènes *stx* et en aval de l'antiterminateur [178]. Par conséquent, les mécanismes de régulation de l'expression des gènes *stx* pourraient intervenir au niveau de ces 2 promoteurs

par une régulation directe du promoteur P_{stx} et par une action sur l'induction de la phase lytique du phage.

2.6. Rôle du plasmide pO157 dans la virulence des EHEC

Les déterminants principaux de la virulence chez les souches EHEC sont encodés par le chromosome. Cependant, les plasmides pourraient jouer un rôle important dans la pathogénèse des souches EHEC de sérogroupe O157 et qui causent la maladie. Le plasmide pO157 est retrouvé dans 99 à 100 % d'isolats cliniques humains d'*E. coli* O157:H7 [142,179,180]. Cependant le rôle de pO157 dans la pathogenèse des EHEC n'est pas clairement défini. Certains rapports corrèlent pO157 avec l'activité hémolytique et l'adhérence aux cellules épithéliales, mais l'absence de model humain d'infection empêche le progrès de notre compréhension globale sur la pathogenèse des EHEC reliée au pO157 [181].

Le premier plasmide pO157 séquencé provenait d'une de la souche EDL933 lors d'une éclosion causant des CH aux États-Unis [181]. Ce plasmide de 92-Kb contient des facteurs de virulence tels que l'opéron hémolysine (*ehxCABD*), un système de sécrétion de type II (*etpC-O*), une protéase extracellulaire (*espP*), une catalase-peroxydase (*katP*), et un homologue de *toxB*. L'hémolysine des EHEC (Hly ou Ehx) est une toxine formatrice de pore qui lyse les hématies [182,183]. Bien que le rôle d'Ehx dans la virulence ne soit pas clair, plusieurs investigations suggèrent sa contribution à la maladie. Les hémolysines peuvent s'associer en tant que cargos avec les vésicules de membrane externe (OMVs), ce qui prolonge leur activité [184]. Additionnellement, Ehx a un effet cytotoxique sur les cellules endothéliales ce qui pourrait contribuer au développement de SHU [185]. Récemment il a été démontré que Ehx pourrait être inactivée par la protéase EspP [186]. Parmi les 4 sous-types de sérine protéases EspP, seulement EspPα EspPγ sont actives et sécrétées [187]. EspPα est le plus communs chez les souches STEC O157 corrélées avec la sévérité de la maladie [187]. Les EspP sont des auto-transporteurs avec des multifonctions protéase. Les EspP clivent le facteur V de coagulation, le complément et l'hémolysine des EHEC [186,188,189].

Puttamreddy et al, [190] ont montré que Ehx et EspP sont directement impliquées dans la formation de biofilm chez EDL933 et sont requises pour l'adhérence aux cellules épithéliales, suggérant leurs rôle dans l'interaction avec les tissus *in vivo*.

3. Les amibes, partenaires possibles des EHEC dans l'environnement

3.1. Les Amibes

Les amibes sont un groupe d'organismes appartenant au règne de protistes qui forment un groupe divers d'eucaryotes inférieurs. Ils sont référés aux protozoaires, un groupe de protistes hétérotrophiques. Les amibes font partie des protozoaires motiles par des pseudopodes. Ils consomment des bactéries, levures, algues et autres protistes par phagocytose et engloutissent des solutés et particules de nourriture par pinocytose [191-193]. Elles mangent plusieurs types de proies mais ont tendance à être sélectives envers certains types d'aliments [194]. Les amibes se reproduisent asexuellement par scissiparité. Leur cycle de vie se compose de 2 stades: le stade à l'état trophozoïte végétatif se nourrissant activement de bactéries et le stade à l'état dormant sous forme de kyste à double paroi à base de cellulose. Le kyste se forme lorsque les conditions sont défavorables telles que des conditions extrêmes de température, pH, salinité, et le manque de nourriture. Pendant le processus d'enkystement, les excédents de nourriture, d'eau et de particules sont expulsés et le trophozoïte se condense en un «pre-kyste». Ce prekyste mûrit ensuite en un kyste à double paroi résistant et protecteur contre l'environnement hostile. Les niveaux cellulaires d'ARN, de protéines, de triglycérides et du glycogène baissent en réduisant considérablement le volume et le poids sec cellulaire. Pendant leur stade de kyste, les amibes sont protégées de la dessiccation, de la famine, des désinfectants et agents antimicrobiens, d'anoxie, de pH et températures extrêmes et des rayons UV. Ces kystes peuvent devenir aéroportés et facilement propagés dans l'environnement et possèdent des ostioles qui sont des pores détecteurs de changements environnementaux. Lorsque les conditions sont à nouveau favorables, le trophozoite ré-émerge à partir du kyste laissant derrière la coque extérieure et poursuit son cycle de vie [191-193,195,196].

3.2. Les *Acanthamoebae*

Les protistes appartenant au genre *Acanthamoeba* sont d'importants prédateurs qui peuvent contrôler et modifier les communautés microbiennes où ils résident [197,198]. Les espèces d'*Acanthamoebae* sont caractérisées par des structures épineuses ou «acanthopodes» sur la

surface du trophozoite permettant l'adhérence aux surfaces, le mouvement, et la capture de proies [191]. Les *Acanthamoebae* sont omniprésents dans les habitats écologiques. Ils sont retrouvés dans l'eau douce, l'eau saumâtre, l'eau de mer, les eaux usées, la plage de sable, l'air, la poussière, le compost, les légumes et la plupart des sols. Dans les maisons, les *Acanthamoebae* ont été isolées à partir des pots de fleurs, des aquariums, des humidificateurs, des robinets d'eau, des cupules et solutions pour lentilles de contact, des piscines, de l'eau embouteillée et des renvois d'évier [191,193,197]. Elles ont été isolées aussi à partir des cavités humaines; pharynx, tissu pulmonaire, lésions cutanées, biopsies cornée, et liquide céphalo-rachidien [191-193]. Certaines *Acanthamoebae* sont des pathogènes humains qui provoquent des infections oculaires telles que kératite amibienne et causent l'encéphalite granulomateuse amibienne chez les sujets immunodéprimés [192,193].

Les *Acanthamoebae* peuvent aussi être cultivées de façon xénique (avec bactéries) en culture sur agar non-nutritif supplémenté de bactéries vivantes ou tuées. Cependant dans les protocoles expérimentaux, les amibes sont souvent cultivées de façon axénique (en absence de bactérie) mais en milieu de culture liquide, le plus souvent, du PYG (Peptone Yeast Glucose) [194].

3.3. Les *Acanthamoebae* et les bactéries endosymbiotiques

De nombreuses espèces protozoaires, dont de nombreuses espèces d'amibes, peuvent héberger une grande variété de bactéries endosymbiotiques, y compris des pathogènes humains. Ces bactéries sont phagocytées mais parfois non digérées [192,193,199-201]. La pression de prédation, les environnements difficiles, ainsi que la pénurie des nutriments ont façonné des stratégies de défense complexes permettant à certaines bactéries de survivre et parfois se répliquer à l'intérieur des amibes [192,193,199-201]. À l'intérieur des amibes, ces bactéries sont protégées de la dessiccation, des agents antimicrobiens et produits chimiques et des conditions atmosphériques [192,199,200]. Certaines bactéries peuvent même résider à l'intérieur des kystes ce qui les protègent davantage contre les conditions environnementales, permettant leur propagation à de nouveaux habitats notamment via les kystes aéroportés [193,200,202-204].

On estime que les amibes agissent comme des réservoirs de certains pathogènes bactériens leur permettant à de persister dans les conditions défavorables et facilitant leur dispersion dans l'environnement, ce qui augmenterait la transmission des maladies [199]. On ne sait pas si les amibes bénéficient de cette association, mais elles peuvent acquérir des éléments nutritifs [194]. Il a également été rapporté que certaines bactéries endosymbiotiques peuvent affecter la motilité amibienne [205]. On trouve trois scénarios possibles d'endosymbiose: i) Une multiplication intracellulaire de bactéries finissant par la lyse d'amibes, par exemple *Legionella* spp, *Salmonella* spp et *Campylobacter jejuni* [206-210]; ii) Une multiplication bactérienne intracellulaire mais ne causant pas de lyse cellulaire, telles que (*Vibrio cholerae* et *Shigella* spp [202,211]; iii) Certaines bactéries telles que *Mycobacterium* spp, survivent intra-cellulairement sans réplication [212]. La symbiose avec les agents pathogènes endommage souvent l'amibe ou entraine sa mort, alors que les organismes non pathogènes causent peu ou pas d'effets néfastes pour l'amibe. De plus, à l'intérieur des amibes, certaines bactéries intracellulaires, notamment *Legionella* spp. et *Chlamydia* spp., peuvent s'échanger des gènes [213]. Les amibes peuvent également favoriser la croissance de certains pathogènes par la sécrétion de facteurs extracellulaires. Il a été signalé que *Vibrio parahaemolyticus* pouvait éviter sa phagocytose par *Acanthamoeba castellanii* mais était capable de survivre en culture plus longtemps en présence de cette amibe qu'en son absence [214]. Des résultats similaires ont été rapportés pour *Listeria monocytogenes* qui a été capable d'utiliser des matériaux libérés par l'amibe pour survivre extra-cellulairement [215].

Les amibes sont considérées comme des cellules phagocytaires avec des caractéristiques similaires aux phagocytes de mammifères tels que les macrophages. Le développement de stratégies permettant la survie intra-amibe pourrait être à l'origine de l'invasion de macrophages par les bactéries pathogènes associées aux amibes [199]. Cela permet de décrire les amibes comme "terrain d'entraînement" des agents pathogènes, parce que les bactéries ont besoin d'éviter/survivre à l'internalisation par les amibes de manière similaire face à la réponse immunitaire humaine [194,216]. Cirillo *et al.*, ont démontré que la culture de *L. pneumophila* dans *A. castellanii* augmente la virulence des bactéries et leur réplication dans les monocytes [206]. De manière similaire, il a été reporté une augmentation d'expression des gènes de virulence de *Salmonella* à l'intérieur d'*Acanthamoeba rhysodes* [208].

3.4. Association des EHEC avec les protozoaires

Des études initiales ont investigué le rôle des espèces protozoaires dans la survie environnementale et la pathogénicité des *Legionella spp* [217]. Ces interactions ont ouvert la voie à d'autres investigations vastes où d'autres espèces bactériennes peuvent survivre à l'intérieur de protozoaires, principalement des amibes (Table I). Chaque pathogène interagit avec les protozoaires de manières différentes, et il est donc important de recenser les données de littérature sur les interactions de tout pathogène bactérien avec les amibes, pour avoir une meilleure idée de ce qui se passe lors de l'interaction entre *A. castellanii* (notre modèle protozoaire d'interaction hôte-pathogène) avec les EHEC (notre objet d'étude dans ce projet).

Table I. Compilation d'espèces de protozoaires et les bactéries qu'ils hébergent et/ou protègent

Hôte protozoaire	Bactéries			Références
Acanthamoeba astronyxis	*Burkholderia pseudomallei*			[201]
Acanthamoeba castellanii	*Acinetobacter baumannii* *Acinetobacter haemolyticus* *Acinetobacter johnsonii* *Acinetobacter junii* *Acinetobacter lwoffii* *Acinetobacter radioresistens* *Aeromonas caviae,* *Aeromonas hydrophila* *Aeromonas veronii* *Bacillus licheniformis* *Bacillus pumilus* *Campylobacter jejuni* *Campylobacter lari* *Campylobacter coli* *Campylobacter hyointestinalis*	*Chlamydia pnuemoniae* *Citrobacter freundii* *Coxiella burnetii* *Enterobacter cloaca* ***Escherichia coli (O157)*** *Francisella tularensis* *Helicobacter pylori* *Klebsiella oxytoca* *Klebsiella pneumonia* *Legionella anisa* *Legionella pneumophila* *Listeria ivanovii* *Listeria monocytogenes* *Listeria seeligeri* *Listeria welshimeri*	*Mycobacterium avium* *Mycobacterium bovis* *Parachlamydia acanthamoeba* *Porphyromonas gingivalis* *Protochlamydia amoebophila* *Salmonella spp* *Shigella dyseneriae* *Shigella sonnei* *Simkania negevensis* *Vibrio cholera* *Vibrio mimicus* *Waddia chondrophila* *Yersinia spp*	[198,201,203,206,207,211,214,218-234]
Acanthamoeba culbertoni	*Legionella feeleii, Legionella pneumophila*			[201]
Acanthamoeba griffin	*Legionella pneumophila*			
Acanthamoeba lenticulata				
Acanthamoeba palestinensis				
Acanthamoeba rhysodes	*Campylobacter lari, Campylobacter coli, Campylobacter hyointestinalis, Campylobacter rhysodes, Salmonella spp.*			[208,209, 229]
Acanthamoeba royreba	*Legionella pneumophila*			[201]
Acanthamoeba polyphaga	*Achromobacter xylosoxidans* *Acinetobacter baumannii* *Acinetobacter calcoaceticus* *Acinetobacter junii* *Actinobacterium spp* *Aeromonas eucrenophila* *Aeromonas hydrophila* *Aeromonas salmonicida* *Afipia felis* *Agrobacterium tumefaciens* *Bacillus cereus* *Bacillus subtilis* *Bradyrhizobium japonicum* *Brevundimonas diminuta* *Brevundimonas vesicularis* *Burkholderia cepacia* *Caedibacter acanthamoeba* *Camamonas aquatic* *Campylobacter coli* *Campylobacter hyointestinalis* *Campylobacter jejuni* *Campylobacter lari* *Chromobacteririum violaceum*	*Enterococcus faecalis* ***Escherichia coli (O157)*** *Flavobacterium johnsoniae* *Flexibacter Canadensis* *Hafnia alvei* *Klebsiella oxytoca,* *Klebsiella pneumonia* *Klebsiella variicola,* *Kluyvera cryocrescens,* *Legionella anisa,* *Legionella drancourtii,* *Legionella pneumophila,* *Legionella rubrilucens,* *Listeria monocytogenes,* *Methylobacterium mesophilicum* *Microbacterium oxydans* *Morganella morganii* *Mycobacterium abscessus* *Mycobacterium avium,* *Mycobacterium paratuberculosis* *Mycobacterium chelonae,* *Mycobacterium fortuitum,* *Mycobacterium gordonae*	*Mycobacterium szulgai* *Mycobacteria ulcerans* *Mycobacterium xenopi* *Mycobacteria spp.* *Ochrobactrum anthropic* *Panteoea agglomerans* *Pasteurella multocida* *Providencia alcalifaciens* *Pseudomonas alcaligenes* *Pseudomonas aeruginosa* *Pseudomonas fulva* *Pseudomonas mendocina* *Pseudomonas plecoglossicida* *Pseudomonas putida* *Rickettsia bellii* *Salmonella enterica* *Sarcobium lyticum* *Serratia plymuthica* *Rhodococcus equi* *Rothia dentocariosa* *Sphingomonas spp.* *Sphingobacterium spp.* *Sphingobacterium multivorum*	[198,201,210,215,225,229,234-245]

	Chryseobacterium meningosepticum *Delftia spp* *Delftitia acidovorans,* *Delftia tsuruhatensis,* *Enterobacter aerogenes,* *Enterobacter amnigenus* *Enterobacter cancerogenus* *Enterobacter cloaca*	*Mycobacterium kansasii* *Mycobacterium malmoense,* *Mycobacterium marinum* *Mycobacterium mucogenicum* *Mycobacterium peregrinum* *Mycobacterium porcinum* *Mycobacterium simiae* *Mycobacterium smegmatis*	*Staphylococcus aureus* *Staphylococcus pasteuri* *Stenotropomonas maltophilia* *Streptococcus pneumonia* *Vibrio cholera* *Yersinia enterocolitica*	
Acanthamoeba spp.	*Afipia broomae* *Afipia felis* *Amoebophilis asiaticus* *Bacillus anthracis* *Bosea spp.* *Bradyrhizobium japonicum* *Burkholderia cepacia* *Burkholderia pickettii* *Burkholderia pseudomallei Caedobacter acanthamoeba* *Chlamydophila pneumonia* *Coxiella burnetii* *Cytophaga spp.* ***Escherichia coli*** *Flavobacterium spp.* *Francisella tularensis* *Helicobacter spp.* *Helicobacter pylori* *Legionella spp.*	*Legionella pneumophila* *Legionalla anisa* *Legionella lytica* *Legionella dumoffii* *Legionella fallonii* *Legionella rowbothamii* *Legionella drozanskii* *Legionella drancourtii* *Legionella longbeachae* *Legionella-Like* *Listeria monocytogenes* *Mezorhiobium amorphae* *Molibuncus curtsii* *Mycobacterium leprae* *Mycobacterium avium* *Mycobacterium marinum* *Mycobacterium ulcerans* *Mycobacterium simiae* *Mycobacterium phlei*	*Mycobacterium fortuitum* *Mycobacterium smegmatis* *Mycobacterium bovis* *Mycobacterium tuberculosis* *Odyssella thessalonicensis* *Paracaedibacter acanthamoeba* *Paracaedibacter symbiosus* *Parachlamydia acanthamoeba* *Procabacter acanthamoeba* *Procabacter spp.* *Pseudomonas aeruginosa* *Ralstonia pickettii* *Riskettsia-like* *Salmonella enterica* *Simkania negevensis* *Staphylococcus aureus* *Staphylococcus epidermidis* *Vibrio cholera* *Waddlia chondophila*	[198,201,204,215,234,246-252]
Balamuthia mandrillaris	*Legionella pneumophila*			[201]
Dictyostelium discoideum	*Legionella pneumophila, Legionella – like amoebal pathogens, Mycobacterium avium, Mycobacterium tuberculosis, Mycobacterium marinum, Psuedomonas aeruginosa, Salmonella, Vibrio cholerae*			[201,253-259]
Echinamoeba exundans	*Legionella pneumophila*			[201]
Entamoeba dispar	***Escherichia coli**, Shigella spp.*			[260]
Hartmannella cantabrigiensis	*Legionella pneumophila*			[201]
Hartmannella vermiformis	*Campylobacter lari, Campylobacter coli, Campylobacter hyointestinalis, Campylobacter jejuni, **Escherichia coli**, Legionella-like ameobal pathogens, Neochlamydia hartmannellae, Sarcobium lyticum*			[198,200,201,203,206,229]
Hartmannella spp	*Alcaligenes spp., Bacillus spp., Legionella spp., Legionella pneumophila, Pseudomonas spp*			[198,204]
Naegleria australiensis	*Legionella pneumophila*			[201]
Naegleria fowleri	*Bacillus licheniformis, Legionella pneumophila*			
Naegleria gruberi	*Legionella pneumophila, Legionella spp., Vibrio cholera*			[198,201]
Naegleria Iovaniensis	*Legionella pneumophila*			[201]
Naegleria jadini				
Platyamoeba placida				
Saccamoeba spp	*Ehrlichia-like, Legionella pneumophila*			[198,201]
***Tetrahymena pyriformis* (ciliate)**	*Aeromonas salmonicida, Campylobacter jejuni, Campylobacter coli, Campylobacter lari, Campylobacter hyointestinalis, Citrobacter freundii, Edwardsiella tarda, Enterobacter cloaca, **Escherichia coli (O157)**, Klebsiella oxytoca, Klebsiella pneumonia, Legionella gormi, Legionella anisa, Legionella feeleii, Legionella hackeliae, Legionella longbeachae, Legionella oakridgensis, Legionella pneumophila, Listeria monocytogenes Salmonella enterica, Salmonella Typhimurium, Shigella sonneii, Yersinia enterocolitica*			[201,204,218,220,229,251,261-265]
Vahlkampfia jugosa	*Legionella pneumophila*			[201]
Vexillifera spp				
Willaertia spp				

Bien que les *E. coli* soient une source de nourriture commune pour les amibes [192,251], des souches virulentes se sont révélées capables de survivre dans *A. polyphaga* et *A. castellanii* [223]. Dans une étude impliquant *A. polyphaga*, EHEC a survécu plus de 35 jours à 25 °C en co-culture avec les amibes, contre 10 jours en leur absence. Les bactéries ont

d'abords été observées à l'intérieur des vacuoles avant d'être expulsées [245]. Des cellules *E. coli* ont été aussi observées dans les kystes amibiens, et il a été reporté que les cellules d'*E. coli* O157 subissaient un taux de digestion non-suffisant pour toutes les éliminées de l'intérieur des amibes [245]. Il a été rapporté que *E. coli* K1 (souche invasive avec capsule de sérogroupe 1) pouvait s'associer et envahir *A. castellanii* [223]. De plus, *E. coli* K1 pouvait se multiplier à l'intérieur de l'amibe, alors que *E. coli* K12 était rapidement digérée et éliminée par l'amibe [223].

Contrairement à la souche *E. coli* K1, les *E. coli* O157 sont, à la base, considérée comme des souches non-invasives. De plus, les *E. coli* O157 expriment des facteurs de virulence comme les Stx, connus pour leur capacité de lyse de cellules hôtes. Cela rend intéressant l'investigation de l'éventuelle association des EHEC et *A. castellanii*. Par ailleurs les EHEC peuvent s'associer et survivre dans des protozoaires non-amibiens tel que les ciliées. Ceci est discuté dans l'article de revue 1 (voir chapitre I; Table I, article de revue #1 et discussion générale).

4. Contexte de recherche et objectifs de ce projet

Dans les milieux de l'environnement où se retrouvent les EHEC, elles rencontrent potentiellement des protozoaires tels que l'amibe *A. castellanii*. Dans ces milieux le Pi et présent en quantités infimes. Notre hypothèse est que les EHEC s'associeraient avec ou survivraient à l'intérieur de cette amibe. Leur milieu de rencontre étant pauvre en Pi induit l'expression du régulon Pho ce qui favoriserait la persistance des EHEC dans ces conditions. De plus la réponse à la carence en Pi induirait, de manière Pho-dépendant ou indépendante, des changements génotypiques et/ou phénotypiques de virulence des EHEC. La formation de biofilm et la motilité sont deux phénotypes associés au pouvoir infectieux des EHEC. Ces 2 phénotypes pourraient être aussi influencés par la carence en Pi et/ou l'expression du régulon.

Les l'objectifs de ce projet de thèse sont:

i. Vérifier l'hypothèse d'association des EHEC avec *A. castellanii* et de mesurer la survie intra-amibe en évaluant l'impact du régulon Pho, de Stx et SST3 dans ces phénotypes. Nous nous sommes fixé l'objectif de mesurer *in vitro* la cultivabilité et viabilité à long et court-terme des EHEC (la souche sauvage EDL933 et ses mutants, Δ*pst*, Δ*phoB*, Δ*stx* et Δ*escN*) en co-culture avec *A. castellanii,* et leurs survies au test de protection à la gentamicine.

ii. Analyser la transcription globale du génome de la souche EDL933 en réponse à la carence en Pi et aussi en réponse à l'absence du régulateur PhoB. Ensuite, vérifier la variation d'expression des gènes de virulence par qRT-PCR et par fusion génomique avec des gènes rapporteurs. L'effet direct ou indirect du PhoB sur l'expression des gènes de virulence est testé *in vitro* par tests de retard sur gel (EMSA). Les réponses phénotypiques de virulence sont testées en mesurant l'adhésion bactérienne aux cellules HeLa, la sécrétion d'effecteurs du SST3 ainsi que la production et sécrétion des Stx.

iii. Caractériser la formation de biofilm et mobilité des EHEC en fonction de l'expression du régulon Pho et identifier les gènes Pi-dépendants et/ou PhoB-dépendants qui sont impliqués dans le changement de ces 2 phénotypes

5. Article de revue # 1. «L'habitat écologique et la transmission d'*Escherichia coli* O157:H7»

Objectifs

Dans cette revue, nous avons fait une mise à jour des connaissances sur la survie d'*E. coli* O157 dans l'environnement et nous avons discuté les différents facteurs extrinsèque et intrinsèque qui permettraient au EHEC de s'adapter et persister dans l'écosystème.

Contribution de l'étudiant

Étant le premier auteur de cette publication, j'ai écrit près de 90% de l'article. J'ai coordonné et assemblé les corrections de mes directeurs, et soumis le manuscrit au journal FEMS Microbiology Letters. Judith Paquin-Veillette m'a aidé à réfléchir à la conception de la figure présentée dans cet article.

The ecological habitat and transmission of *Escherichia coli* O157:H7

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Abstract

Since its first description in 1982, the zoonotic life-threatening Shiga toxin-producing *Escherichia coli* O157:H7 has emerged as an important food- and water-borne pathogen that causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome in humans. In the last decade, increases in *E. coli* O157:H7 outbreaks were associated with environmental contamination in water and through fresh produce such as green leaves or vegetables. Both intrinsic (genetic adaptation) and extrinsic factors may contribute and help *E. coli* O157:H7 to survive in adverse environments. This makes it even more difficult to detect and monitor food and water safety for public health surveillance. *E. coli* O157:H7 has evolved behaviors and strategies to persist in the environment.

Keywords. *E. coli* O157:H7; ecology; environment; water; survival; free-living protozoa.

Running title: The ecology of *E. coli* O157:H7

Introduction

Enterohemorrhagic *E. coli* (EHEC), in particular serotype O157:H7, is a highly pathogenic subset of Shiga toxin-producing *E. coli* (STEC), that causes gastrointestinal illnesses ranging from aqueous and bloody diarrhea to hemorrhagic colitis in humans [266,267]. Hemolytic-uremic syndrome (HUS) is a potentially life-threatening complication that can arise from STEC infection. The production of Shiga toxins (Stx) is a key factor contributing to the development of HUS [268]. In addition to Stx, a type III protein secretion system (T3SS), through which the pathogen translocates effector proteins into host cells, causing attaching and effacing (A/E) lesions [269]. The genes required for A/E lesions are encoded within a chromosomal pathogenicity island named the *Locus of Enterocyte Effacement* (LEE) [270]. The LEE encodes T3SS, an adhesin (the intimin Eae) and its receptor (Tir) required for intimate adherence to epithelial cells, and effector proteins translocated through the T3SS that are injected into the host cell [271]. The genome sequences of O157:H7 strains isolated from the major outbreaks share about 75% of a highly conserved sequence backbone of the *E. coli* chromosome [272,273]. The remaining O157:H7-specific sequences are named O islands, most of which are horizontally transferred and include other virulence genes in addition to *stx* and LEE genes [274].

Cattle are recognized as the main reservoir for *E. coli* O157:H7 resulting in zoonotic transmission by consumption of under-cooked meat or dairy products inadequately pasteurized and contaminated with bovine feces [275,276]. Here we review the established and putative environmental behaviors of *E. coli* O157:H7 and present potential reservoirs and ecological niches where EHEC may persist in the environment.

E. coli O157:H7: an emerging food- and water-borne pathogen

E. coli O157:H7 and other serotypes of the STEC group are naturally acquired infections that have been detected in a wide spectrum of animal species (cattle, sheep, goat, deer, moose, swine, horse, dog, cat, pigeon, chicken, turkey, gull) sometimes even with considerable prevalence [277,278]. In particular, cattle have been identified as major reservoirs of STEC strains that are highly virulent in the human host (e.g., EHEC O157:H7). However, in contrast to the human host, most STEC infections of animals are clinically asymptomatic [279,280]. *E.*

coli O157:H7 infections in humans often occur through consumption of contaminated food products derived from cattle. Analysis of 90 confirmed *E. coli* O157:H7 outbreaks that occurred between 1982 and 2006 in Canada, Great Britain, Ireland, Japan, Scandinavia and USA, indicated that 20% of cases were the result of secondary spread [281]. The authors found that the source of transmission was food and dairy products in 54%, water and environmental in ~10% and animal contact in ~8%. Consumption of any food or beverage contaminated with animal manure/feces can result in disease. For this reason, food sources causing illness secondary to *E. coli* O157:H7 outbreaks have changed in the past several years. Interestingly, fresh greens, fruits and vegetables have become important sources of human infection. In the USA, *E. coli* O157:H7 infections from contaminated fruits and vegetables increased from 11% to 41% from 1998-2007 [282]. Contamination of fresh produce was associated with fecal contamination in agricultural irrigation water or runoff.

Along the same line, the recent enterohemorrhagic *E. coli* strain O104:H4 was implicated in the spring 2011 European outbreak and its possible source was associated with raw vegetables (fenugreek seeds or sprouts) consumed raw or undercooked [283]. It was difficult to establish the link between vehicle, source and cause of this STEC outbreak. This raised the importance of epidemiological and microbiological investigations in food and in the environment.

The global community has been experiencing food-associated outbreaks of non-O157 STEC for nearly two decades. As a result, increasing scientific evidence supports that *E. coli* non-O157 strains have a high prevalence in meat products, and are equally capable of causing severe food borne illness outbreaks [284]. Hussein (2007) reviewed reported levels of non-O157 STEC in whole cattle carcasses, ground beef, retail beef cuts, and sausage and found 1.7–58%, 2.4–30%, 11.4–49.6%, and 17–49.2%, respectively [285]. In table I, the reports of non-O157 STEC in foods, associated with human infections from numerous countries reveal wide variation in prevalence estimates and in the major non-O157 serogroups reported [286-289].

STEC non-O157 are less likely than O157 STEC to cause outbreaks or severe disease and because they are more challenging diagnostically, many non-O157 infections may not be investigated fully and their sources may thus remain undetermined. The incidence of non-

O157 STEC declaration has increased as laboratories got involved in testing for those strains [290-292]. It is possible that the occurrence of non-O157 outbreaks is due to environmental persistence [293].

E. coli O157:H7 outbreaks related to consumption of contaminated water or to the use of surface water for recreational purposes have been reported [294-296]. In 1999, people became sick after drinking contaminated water in Washington County, New York and swimming in contaminated water in Clark County, Washington. The outbreak in Walkerton, Canada (May 2000) related to consumption of drinking water that was contaminated by feces caused 2,300 disease cases [297]. *E. coli* O157:H7 and *Campylobacter jejuni* were identified as the main pathogens responsible for these disease cases, and *E. coli* O157:H7 was responsible for seven deaths [297]. One factor explaining this contamination is the impact of climate. Indeed, surface water-bodies can become contaminated by *E. coli* O157:H7 after a heavy rainfall or snowmelt, causing sewers to overflow or animal feces and manure to mix into surface water [298]. Also, some human-to-human contamination cases were reported due to the presence of *E. coli* O157:H7 in water such as public pools or lakes [298,299]. Persons with diarrhea, especially children after shedding or changing soiled diapers can contaminate recreational waters and infection could occur by bathing and/or swallowing [298-301]. Outbreaks associated with non-O157 STEC strains through water and produce have been documented [302]. Some strains of non-O157 STEC have been reported to survive in untreated well water for several months [303]. Persistence might be underestimated and could be comparable to O157.

How *E. coli* O157:H7 persists in natural ecological niches?

Pathogenic *E. coli* strains can survive in open environments. The ability to use nutrients and to attach to surfaces plays a crucial role in their survival in open environments. *E. coli* O157:H7 is found in soil, manure and irrigation water or contaminated seeds. Also, it may colonize the interior of plants such as radish, lettuce and internal plant compartments [304-306]. This makes it difficult to remove or kill these germs by washing and/or disinfection. Moreover, *E. coli* O157:H7 may be introduced into the food chain by splashing during rainfall or irrigation [307]. Thus, from contaminated consumable products and the transfer to other products during

food processing and packaging, the organism can be disseminated in the food production chain.

E. coli from livestock feces is known to survive on grass pasture for at least 5 months, affording opportunity for *E. coli* O157:H7 to be recycled by animals [308]. Furthermore, the immediate environment of the animal and its feeding and drinking water are important sources of *E. coli* O157:H7 infection of cattle (reviewed in Fairbrother & Nadeau 2006). The risk factors for carriage and infection of cattle are age, weaning, shipping, season, and feed composition and the bacteria's ability to persist in the farm environment for months [309]. Thus *E. coli* O157:H7 represents an underestimated environmental risk.

Factors involved in environmental persistence of *E. coli* O157:H7

Bacteria constitute the most successful form of life in environmental habitats. This is due to the ability to respond to environmental stimuli by phenotypic plasticity. In their ecosystem cycle, bacteria like *E. coli* O157:H7 are subjected to fluctuations in environmental conditions in soils and water. Viability and growth of bacteria depends primarily on availability of essential nutrients including organic carbon, phosphate (P) and nitrogen [310]. However, *E. coli* O157:H7 survives even at low density in oligotrophic environments such as surface water or groundwater that may be used as a raw water source for drinking water. This may be in river water containing low concentrations, 0.1 − 0.7 mg/liter, of organic carbon [311].

The presence of *E. coli* O157:H7 in aquatic environments is the common denominator linking diverse transient habitats and transmission to animals and humans. Therefore, it appears essential to understand the aquatic ecology of *E. coli* O157:H7. This section highlights the adaptation of *E. coli* O157:H7 in aquatic environments and analyzes its survival and growth in sessile biofilm state and unfavorable conditions that support a viable but non-culturable (VBNC) state and free planktonic cells in water that may promote dissemination.

Aquatic *E. coli* O157:H7- A starvation-survival lifestyle

Various studies have reported that survival times of *E. coli* O157:H7 strains in aquatic environments vary importantly, ranging from 2 weeks to over 10 months [312,313]. It is thus important to attempt to identify the factors responsible for its survival rate. Many factors,

individual or combined, may influence the pathogen's survival such as the temperature, the bacterial cell numbers, strain variation, oxidative stress, nutrient availability, and the substrate type or source.

Unlike some Gram-positive bacteria that respond to starvation by producing spores or cysts, *E. coli* O157:H7, a non-sporulating bacterium, might respond more by an altered physiological or metabolic state instead of developing resistant structural modifications. On the other hand, *E. coli* growing under nutrient-sufficient conditions (i.e. animal feces or manure) accumulates reserve carbon sources that can be stored for use in nutrient poor environments [314]. Indeed, when nutrient conditions in aquatic habitats are unfavorable, *E. coli* O157:H7 might reduce cell size thereby increasing its surface/volume ratio and allowing more efficient uptake of poorly available nutrients. This physiological state resulting from an insufficient amount of nutrients is known as the starvation-survival state [315]. In this state, *E. coli* has evolved strategies to acclimate rapidly to surrounding environmental changes. The adaptive response begins by activation of enzymes required to catabolize available nutrients [316]. After that, *E. coli* may increase its production of toxins or antibiotics, to promote killing or invading of other cells in the environment [317,318]. Finally, the bacterium switches to a survival state, which enhances its resistance to many stresses and its ability to remain viable during long periods without nutrients [319].

Carbon and phosphate stress responses of aquatic *E. coli* O157:H7

E. coli O157:H7 may survive and even grow in sterile freshwater at low carbon concentrations [320]. Bacteria respond to specific nutrient stresses by producing transport systems with increased affinities for the nutrients most easily exploited, and then they express transport and metabolic systems for alternative nutrient sources. Thus, these bacteria may be able to escape starvation by more efficient scavenging of a preferred nutrient or by using another, relatively more abundant source.

When *E. coli* O157:H7 is starved or stressed, cells enter into a general stress response phase [310]. At this point, the production of RpoS orchestrates the transcription of a series of overlapping networks of genes responsible for the *E. coli* general stress response [321]. RpoS competes with the housekeeping sigma factors to direct a core RNA polymerase in the

transcription of specific gene subsets, switching on the stress metabolisms to prepare the bacteria to resist multiple environmental stresses including starvation [322]. High osmolarity, fluctuations of temperature, low pH and low growth rate also induce the RpoS response in *E. coli* cells [323-326]. *E. coli* O157:H7 was found to be more acid resistant than generic *E. coli* strains [327]. Acid tolerance varies among strains [328]. Oh *et al*., reported significantly higher tolerance to acetic acid of *E. coli* O157:H7 strains from environmental sources including water and bovine feces as compared with human outbreak-related strains [329]. In addition, it has been shown that *E. coli* O157:H7 *rpoS* deletion impaired expression of genes responsible for stress response including *gadA* (part of glutamate-dependent acid resistance system 2) and *ler* (LEE-encoded regulator) [330].The importance of *rpoS* in aquatic environments is also supported by the decreased survival of *E. coli rpoS* mutant in stationary phase in sea water [331].

Growth and survival of *E. coli* in open environments is often restricted by the availability of nutrients and energy sources. However, in surface waters, viable *E. coli* O157:H7 was detected over a two-month period, in spite of a decline in the cell numbers [332]. Regarding *E. coli* O157:H7, the dynamics of gene expression in a transient habitat such as surface water remain under-explored. Recently, it has been shown that, even as the population of *E. coli* O157:H7 declined, some cells survived in sterile stream water for up to 234 days [333]. In this study, *E. coli* O157:H7 in natural sterile water triggered a stress response metabolism and DNA repair mechanisms indicating that bacteria remained active. However, no variations were reported concerning expression of virulence genes. In contrast, in another study it was found that the gene expression response of *E. coli* O157:H7 to a growth transition in minimal glucose medium triggered expression of genes located on pathogenicity islands and toxin-converting bacteriophages [334]. It is possible that gene expression could vary considerably between adaptations to growth in minimal glucose medium compared to survival in water.

Phosphate is a highly sought after resource. Once used, it is often a limiting nutrient in environments, and its availability may govern the rate of growth of organisms. This is generally true of freshwater environments [335-337]. In most environments, when inorganic phosphate (Pi) availability becomes limiting (< 4 μM), the Pho-regulon is activated [338,339].

Such a global regulatory system permits an optimal adaptive response and the efficient use of phosphate under Pi-limited conditions, which may lead to survival of pathogenic *E. coli* under phosphate-limiting conditions. In addition to playing an important role in virulence, the Pho-regulon may therefore also contribute to persistence of pathogenic *E. coli* in the environment [340]. More recently, Yoshida *et al* identified novel Pho-regulon genes within specific O157-islands that are not localized in the backbone region shared with commensal *E. coli*. They showed that some of those genes are not related to Pi metabolism or utilization. This suggests that in response to environmental Pi stress, the Pho-regulon regulates not only genes involved in Pi homeostasis but also in other functions of *E. coli* O157:H7 [341].

Environmental *E. coli* O157:H7 and biofilm formation

Some strains of *E. coli* O157:H7 form biofilms on both biotic and abiotic surfaces outside the host such as stainless steel, glass, and polystyrene [342-346]. The genetic mechanism of *E. coli* O157:H7 biofilm formation is a complex process and is linked to the production of curli, long polar fimbriae, elements encoded by genes carried by O island OI-1, cellulose and colonic acid [344,347-352]. *E. coli* O157:H7 biofilm formation is also linked to the expression of some virulence genes including gene on the virulence plasmid pO157 [353]. Additionally, intercellular signal molecules, such as autoinducer-2 and indole are also involved in *E. coli* O157:H7 biofilm formation [347,354,355]. *E. coli* O157:H7 uses the T3SS, flagella and the pilus curli to attach and colonize surfaces (plant stomata and internal tissues), which constitute the first step of biofilm formation [282,356,357]. Growth of *E. coli* O157:H7 in protected biofilms proved to be a great advantage in open environments. In diverse habitats, bacteria within biofilms are notably resistant to bacteriophages and to free-living amoeboid predators [358].

E. coli O157:H7 represents a persistent contamination from both the industry sector and throughout its ecological cycle (Fig. 1). The shedding of *E. coli* O157:H7 ranges from 10^2−10^5 CFU/g of feces in cattle [359] and where they can persist and be recycled in the farm environment, soil and water [19,312]. Regardless of whether the water habitat is oligotrophic surface water or groundwater, it should be viewed as an environmental source of *E. coli* O157:H7. Considering all, in their planktonic forms environmental *E. coli* O157:H7 could be

present in a variety of ecosystems, and when nutrient conditions become favorable, phenotypic flexibility allows them to form biofilms. Furthermore, it is now established that the biofilm mode of growth is predominant in aquatic ecosystems, as planktonic populations have been shown to constitute less than 0.1% of the total microbial community.

While there is still no clear link between biofilm formation and the presence or survival of *E. coli* O157:H7 in water, some studies showed that *E. coli* O157:H7 persists in water obtained from bottom-shore sediments [360]. Interestingly, it has been shown that when growing in biofilm, *E. coli* O157:H7 increased its retention and survival in the effluent through a bench-scale sand aquifer system [361]. Also, in slaughter plants, the biofilm mode increases the persistence and acid tolerance of *E. coli* O157:H7 in liquid meat wastes [362]. Furthermore, *E. coli* O157:H7 persists in soils around farms and livestock production and can resist to fumigation. It has been suggested that chemical fumigation that decreases the microbial diversity would favour *E. coli* O157:H7 [363]. Thus, the microbial species diversity participates in the environmental protection of *E. coli* O157:H7.

In these wet or dry surfaces, biofilms could provide an ideal micro-environment for the establishment of syntrophic relationships in which *E. coli* O157:H7 would depend on other bacterial populations to utilize specific substrates, typically for energy production. In fact, it has been shown that non-biofilm-forming *E. coli* O157:H7 strains are retained on solid surfaces associated with biofilms generated by companion strains [364].

Cellular quiescence: A possible mechanism of *E. coli* O157:H7 survival in water

There is little information regarding the behavior and metabolic status of *E. coli* O157 in environmental water sources. However, some survival studies have used culture-based methods that rely on sampling of environmental material, followed by plating on selective media, such as cefixime and potassium tellurite containing sorbitol MacConkey agar [334]. *E. coli* O157:H7 cells in a ‘dormant’ state, also called VBNC, are still alive and demonstrate very low levels of metabolic activity, and are not easily recovered on standard laboratory media [365].

The VBNC state can be triggered by stress conditions in surface water that are imposed by low temperature or toxic metals [366]. However, the occurrence of the VBNC state in enteric bacteria is highly disputed by some reports, while others suggest it does occur in *E. coli* O157:H7 maintained in water and under saline conditions or in cattle manure and slurry [367-371]. These findings showed significantly higher numbers of the organisms by direct microscopic counts when compared to plating on a selective medium, which indicated the prevalence of dormant cells in the total *E. coli* O157 population. The use of bioluminescence such as a *lux* marker system, which indicates the energy status of the cell, provides an alternative way to assess the viability of bacteria including VBNC cells [372].

Aquatic detection and isolation of *E. coli* O157:H7 an epidemiological challenge

E. coli O157:H7 in surface waters constitutes a potential threat to human health through either drinking or ingestion during recreational activities. Currently, water contamination detection is based on standard guidelines that rely on microbial indicator concentrations of thermo-tolerant coliforms and enterococci [373]. Still, there are no established correlations between the prevalence and concentration of these fecal indicators of contamination, and the presence of *E. coli* O157:H7 [374,375]. For this reason, quantitative PCR of virulence genes such as genes *stx* and *eae* represents a more targeted detection method. However, PCR-assay sensitivity can be limited by low *E. coli* O157:H7 abundance in samples (less than 100 cells. mL^{-1}) [376]. Pre-enrichment of samples is used to improve sensitivity and circumvent the limitation of PCR assays. However, further studies are needed to interpret for example, the presence of *stx* genes in water samples. In fact, *stx* genes are carried by the temperate λ–like phage. The presence of *stx* cannot be directly correlated with intact or VBNC STEC as *stx* phages can be released from lysed cells in the environment.

Interestingly, new methods are developed to detect *E. coli* O157:H7 from aquatic environments. Using an anti-O157 antibody modified microfluidic chip permits the specific enrichment of *E. coli* O157 including VBNC [376]. Another method involves the concentration of bacterial cells by filtration of water samples on a low-protein-binding membrane (polyvinylidene difluoride hydrophilic membrane) followed by direct extraction of the total RNA and specific RT-PCR amplification for *rfbE* for O157 antigen and *fliC* for H7 flagellin and then electronic microarray detection of *E. coli* O157:H7 [377].

Interaction with protozoa: a possible persistence strategy for *E. coli* O157:H7

Over the last several decades, the importance of protozoa in soil, sewage and water ecosystems and their role in controlling microbial populations has been widely acknowledged [204]. Protozoa are widely distributed in water, soils and effluents [378]. They are likely to constitute an important environmental reservoir for transmission of *E. coli* O157:H7 and other pathogens. Bacterial pathogens including *Vibrio*, *Legionella*, *Mycobacteria,* enteropathogenic *E. coli,* and the meningitis-causing *E. coli* strain K1 multiply and/or survive within protozoa [204,212,220,223,242,267,379]. *E. coli* O157 and non-O157 strains have been shown to survive within the environmental protozoa *Acanthamoeba polyphaga* and *Acanthamoeba castellanii* [220,245,380].

In addition to enhanced environmental survival, bacterial co-habitation with protozoa could induce adaptive changes in bacteria [220,381]. Furthermore, we recently observed that the co-culture of *E. coli* O157:H7 with *A. castellanii* increased bacterial persistence over 3 weeks [382]. In addition, there was a transient internalization and intracellular survival that increased in an isogenic mutant that did not produce Stx. Carruthers *et al*. showed an increased expression of virulence gene expression such as genes encoding Stx, LEE and non-LEE T3SS effectors when *E. coli* O157 was co-cultured with *A. castellanii* [380]. This suggests that EHEC virulence factors may contribute to persistence and survival during interactions with amoebae. The ability of *E. coli* O157:H7 to enter and invade mammalian cells such as bovine mammary cells as well as also human macrophages was also observed [383-385]. Moreover, it is noticeable that amoebae and human macrophages share morphological and functional similarities; especially in their phagocytic activity and parallel mechanisms in their interactions with many bacterial pathogens [216,386]. Consequently, amoebae have been suggested to be a key step in the evolution of environmental bacteria to become human pathogens. Thus *Acanthamoeba* may provide a useful model to study EHEC pathogenesis and to understand their immune evasion mechanisms.

Lainhart *et al.,* found that Stx-encoding bacteria killed the ciliates protozoa using the holotoxin Stx as an anti-predator weapon [387]. Other studies have shown that the presence of

Stx-encoding prophage augmented the fitness of *E. coli* in co-culture with the ciliate protozoa [388]. These authors found that the ratio of Stx+ to Stx− bacteria increased after 3 days co-culture with *Tetrahymena thermophila* that belong to ciliates protozoa presents in ruminants gut. In contrast, other investigators did not observe any advantage or disadvantages of Stx lysogenic phage with the rumen protozoa [389]. Thus, the contribution of Stx to bacterial survival when facing a protozoan seems to be variable depending on the conditions of the challenge and the protozoan models used in the co-culture assay.

There is growing concern about the survival of pathogens in sewage and waste effluents because it is known that *E. coli* O157:H7 can survive in cattle manure slurry for at least several weeks [390,391]. Protozoa present in these effluents could provide a protective niche for pathogens such as *E. coli* O157:H7 [220]. The role of protozoa in the survival of *E. coli* O157:H7 in the natural environment has been less studied. Soils contaminated with organic matter and sewage waste contain greatly increased numbers of protozoa such as *Acanthamoebae* [378]. It is possible that *E. coli* O157:H7 in soil and slurry could be preyed on by free-living protozoa then serving as vectors for the spread of this pathogen. This is especially true if the bacteria are able to survive within cysts (the resistant forms of amoeba) as has been shown for *L. pneumophila*, *V. cholerae* and *M. avium* [212,392]. Bacteria within amoeba cysts could be dispersed by aerosol transmission. Grazing cattle would ingest protozoa in silage and grass, and the ingested protozoa containing bacterial pathogens such as *E. coli* O157 could also be a route of transmission to cattle.

Different species belonging to the 3 protozoan groups (flagellates, ciliates and amoebae) have been isolated from fresh green products found in the supermarket (i.e. spinach and lettuce). Gourabathini *et al.,* demonstrated that those protozoa can ingest bacterial pathogen including *E. coli* O157:H7 and *S. enterica, and* then produce vesicles containing intact bacteria [393]. Thus, the presence of protozoa on leafy vegetables and their sequestration of enteric bacteria in vesicles indicate that they may play an important role in the ecology of *E. coli* O157:H7 on fresh green products.

Conclusion

In Fig. 1 we present a global view of the ecology and potential source reservoirs of *E. coli* O157:H7. This pathogen has shown the ability to survive in many adverse conditions. In addition to its capacity to cause infection to humans through consumption of contaminated foods, *E. coli* O157:H7 is able to survive in water, making this pathogenic bacterium an environmental threat to humans. The bacteria may enter a starvation and survival state allowing it to adapt and persist in low nutrient environments such as water. Furthermore, *E. coli* O157:H7 has been shown to survive within environmental protozoa; this could contribute to its persistence. However, the distribution of this bacterium in the environment and the increasing reports of different routes of transmission make it difficult to set up efficient strategies to prevent contamination. There is a need to include environmental monitoring as a surveillance method for EHEC where such infections are problematic worldwide. Information collection and sampling methods should be standardized to more clearly understand the ecology of this environmental pathogen. This could lead to rational development of preventative measures to limit its presence in environments that may lead to increased risk for contamination of water, soil, and animals; and transmission and infection of humans.

Conflict of interest statement

The authors declare no conflict of interest.

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Table I. Incidence of STEC and the % of non-O157 in food related to human infections in different area around the world

Country/ Geographic area	EIR[1] of STEC	% of STEC Non-O157	Major non-O157 serogroups[3]	References
Australia	0.4	~ 42	O26, O111	[394]
European Union[2]	1.1	~ 48	O26, O91, O103, O111, O113,O128, O145, O146	[284]
Canada	3.0-6.0	<15	O26, O91, O103, O111, O121	[395,396]
Japan	2.0-3.0	~ 40	O26, O111, O121, O103, O145	[397,398]
Argentina	10.4-12.2	~ 40	O8, O26, O113, O145, O174	[399,400]
USA	1.04-1.2	~ 30	O26, O45, O103, O111, O121, O145	[18,401]

[1] EIR= Estimated Incidence Rate

[2] Data based on 25 reporting countries from the European Union (EU)

[3] Major Non-O157 serogroups

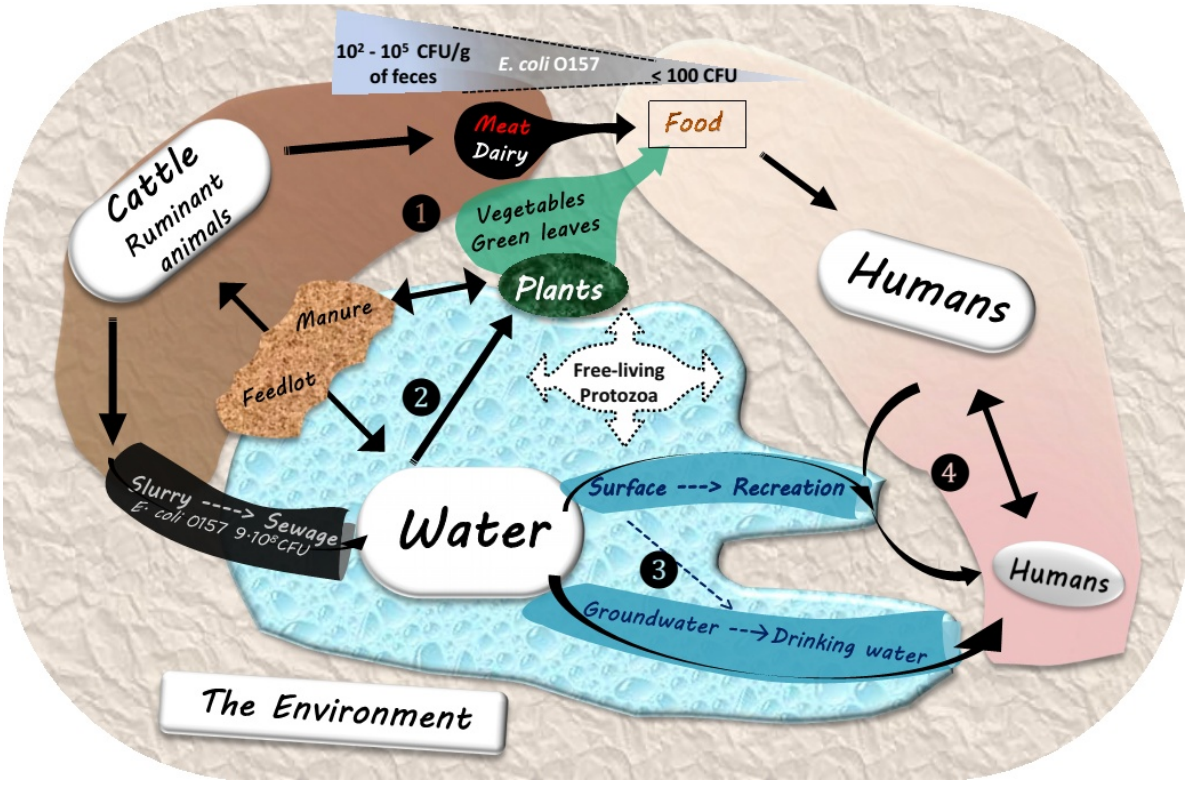

Figure 1. Illustration depicting the ecological habitat and transmission of *E. coli* O157:H7 in a global ecosystem

1. On-farm cattle are the main reservoir for *E .coli* O157:H7 and may contaminate its immediate environment (feedlot), and sewage through cattle manure. Consumption of contaminated animal products (meat and dairy) is the main route of transmission to humans. Also, *E .coli* O157:H7 animal feces (10^2–10^5 CFU/g), through the use of manure, may contaminate plants, allowing the pathogen to also enter the food chain.
2. Water can be contaminated by the animals' immediate environment and manure deposited on lands, sometimes after a heavy rainfall. Furthermore, soils contaminated with sewage waste and overflowing of sewers are causes of contaminated water. This route may then lead to contamination of plants. Also, soils and water containing the free-living protozoa, may serve as vectors for *E. coli* O157:H7.
3. Contaminated surface water bodies are a source of human infection through recreational water activities, while groundwater is the raw water source for human consumption.
4. Human-to-human oro-fecal transmission is another potential route of *E. coli* O157:H7 infection. The human-to-human transmission could occur directly or indirectly through contamination of water by infected humans in recreational waters.

6. Article de revue # 2. «La relation entre la régulation génétique de l'homéostasie du phosphate et la virulence bactérienne»

Objectifs

En 2011, notre groupe a publié une revue traitant du rôle du régulon Pho dans la pathogenèse des *E. coli* pathogènes [340]. Dans la présente revue, nous avons mis à jour et élargi les connaissances sur la relation entre la régulation génétique de l'homéostasie du phosphate et la virulence bactérienne en incluant principalement les données de littérature concernant les pathogènes *Vibrio cholerae*, *Pseudomonas* spp., et *E. coli*.

Contribution de l'étudiant

L'éditeur d'une édition spéciale du journal Virulence à inviter Dr Charles Dozois a partagé ses connaissances en régulation génétique liée à la virulence bactérienne. Mon co-directeur C. Dozois et ma directrice J. Harel m'ont donc chargé de recenser les données de la littérature et d'écrire cet article. J'ai aussi coordonné et assemblé les corrections de mes directeurs, et soumis ce manuscrit au journal Virulence.

Interplay between genetic regulation of phosphate homeostasis and bacterial virulence

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Abstract

Bacterial pathogens, including those of humans, animals and plants, encounter phosphate (Pi)-limiting or Pi-rich environments in the host, depending on the site of infection. The environmental Pi-concentration results in modulation of expression of the Pho regulon that allows bacteria to regulate phosphate assimilation pathways accordingly. In many cases, modulation of Pho regulon expression also results in concomitant changes in virulence phenotypes. . Under Pi-limiting conditions, bacteria use the transcriptional-response regulator PhoB to translate the Pi starvation signal sensed by the bacterium into gene activation or repression. This regulator is employed not only for the maintenance of bacterial Pi homeostasis but also to differentially regulate virulence. The Pho regulon is therefore not only a regulatory circuit of phosphate homeostasis but also plays an important adaptive role in stress response and bacterial virulence. Here we focus on recent findings regarding the mechanisms of gene regulation that underlie the virulence responses to Pi stress in *Vibrio cholerae*, *Pseudomonas spp*. and pathogenic *E. coli*.

Keywords. Phosphate-limitation, Pho regulon, PhoB, Pst, virulence, gene regulation, bacterial pathogens, *Vibrio cholerae*, *Pseudomonas spp*, *Escherichia coli* (*E. coli*).

Introduction

Phosphate (P) is the sixth most abundant inorganic element in living organisms. Its primary biological importance in bacteria is as a component of nucleotides, which serve as energy storage within cells (ATP) or when linked together to form the nucleic acids DNA and RNA. P is also found in membrane phospholipids and required for energy metabolism. It's incorporated into proteins during post-translational modifications, particularly as a regulatory device such as employed during signal transduction of bacterial two-component systems (TCS). For bacteria, the primary sources of P are inorganic phosphate (Pi), pyrophosphate and metaphosphate. They are acquired by the P-specific transport system (Pst) and low Pi-affinity transporter (Pit). Secondary P sources used by bacteria are phosphonates and organophosphates (i.e. Glycerol-3-P) that require separate transporters (Ugp and Phn systems) and enzymatic hydrolysis by alkaline phosphatase (PhoA).

In this review, we focus on recent findings in the mechanisms of gene regulation that underlie the virulence responses to Pi stress in *Vibrio cholerae*, *Pseudomonas spp.* and pathogenic *E. coli*. We also briefly discuss the relationship between Pi regulation and bacterial virulence in additional to non-enteric pathogens.

The Pho regulon and Pi availability

TCSs are signal transduction pathways commonly used by prokaryotes to sense and adapt to stimuli in the environment. As many as 50 different TCSs may exist in a single bacterium such as *E. coli* [402]. These systems are characterized by a sensor histidine-kinase (HK) that undergoes auto phosphorylation on a histidine residue. This phosphoryl group is then transferred to an aspartate residue of a response regulator protein that usually acts as a transcriptional activator (Figure 1). In *E. coli*, there are at least 40 gene members of the Pho regulon that are primarily involved in Pi assimilation and metabolism [403-405]. The core members of the Pho regulon comprise 9 transcriptional units: *eda, phnCDEFGHIJKLMNOP, phoA, phoBR, phoE, phoH, psiE, pstSCAB-phoU,* and *ugpBAECQ*. These units encode proteins which ensure Pi homeostasis including the PhoB-R TCS, alkaline phosphatase and transporters of P, carbon-P, glycerol-3-P and phosphonates. Transcription of genes within the Pho regulon is regulated in response to extracellular Pi concentrations via the TCS PhoB-R.

The Pst system also plays an important role in Pi homeostasis. It has two related but distinct functions: high-affinity uptake of Pi (in low Pi conditions= Pho ON) and the maintainance of Pho regulon expression at basal level (in high Pi conditions= Pho OFF) [406]. The *pst* operon (*pstSCAB–phoU*) encodes a transport complex belonging to the ABC transporter superfamily. The periplasmic protein PstS binds Pi that is able to freely diffuse across the Gram-negative outer membrane, whereas PstA and PstC are membrane channel-forming proteins. The ATPase PstB provides the energy for translocation and interacts with PstA and PstC [407]. PhoU has no evident role in Pi transport, but is required for Pho regulon expression.

During Pi limitation, the HK PhoR phosphorylates PhoB (PhoB~P). PhoB~P is then able to activate the Pho regulon by binding to a consensus Pho box sequence within the promoters of Pho regulon genes [404]. The Pho boxes are formed by direct repeat units consisting of 11 nucleotides, where the first 7 nucleotides are well conserved (consensus sequence GTTCACC). Conversely, when Pi is abundant, the Pho regulon is not induced (OFF state= basal expression), which is mediated by an interaction between the Pst-PhoU complex and PhoR, that prevents PhoR-mediated phosphorylation of PhoB [408]. In these high Pi conditions, PhoB is maintained in non-phosphorylated form by PhoR phosphatase activity, and expression of the Pst system becomes deactivated while the expression of the Pit system becomes de-repressed. Pit does not require chemical energy to drive Pi transport when it is abundant, but when conditions are limiting, the cell uses ATP to scavenge Pi and similar P-containing compounds from the environment via the Pst system [409].

PhoB is a typical winged-helix response regulator that upon aspartyl phosphorylation forms a dimer, which binds in head-to-tail arrangement to DNA sequences upstream of Pho regulon genes to recruit RNA polymerase (RNAP) [410-413] (Figure 1). PhoB~P interacts specifically with the σ^{70} subunit of RNAP, and this interaction facilitates the entry of the RNA polymerase into the *pho* promoters for transcription initiation [414]. In most cases PhoB~P acts as a positive transcriptional factor, but there are a few promoters in which PhoB works as a repressor. This is the case for *hrdA* and *rpoZ* encoding respectively the σ-factor and ω-factor of RNAP in *Streptomyces* species [415,416]. This repression is due to promoter occlusion effect when PhoB~P binds to the -10 to +1 region of the promoter thus interfering with RNAP binding. Another example is PhoB~P repressing *tcpPH* by binding to a Pho-box located between the promoter and the binding sites of AphA and AphB, positives regulators (Figure

2). In this case, the repression has been explained by possible disruption of RNAP interaction with other transcriptional activators [417].

In certain circumstances, PhoB is activated in response to other environmental signals through cross-activation non-partner HK proteins. Indeed, in addition to the well- known CreC, it has been shown that 5 other non-partner HK (ArcB, KdpD, QseC, BaeS and VanS) could also activate PhoB [418,419]. Within a genetically homogeneous cell population, bacteria evolved strategies that display a stochastic character of gene expression in a cross-regulation network of TCSs. This would allow for non-genetic diversity, and thereby a rapid adaption to different environments [420].

Mechanisms by which PhoB controls the virulence of *Vibrio cholerae*

PhoB-mediated genetic regulation of *V. cholerae* toxicity and colonization

In order to cause the cholera diarrhoeal disease, *V. cholerae* induces expression of genes encoding the colonization factor toxin-co-regulated pilus (TCP) and the ADP ribosylating cholera toxin (CT) [421-423]. Dependent on a transcriptional cascade, this is triggered by a pair of independent activators, AphA and AphB (Figure 2A). These 2 activators cooperatively induce the *tcpPH* operon encoding TcpP/H, a transcriptional activator of *toxT* expression.ToxT in turn increases gene transcription of both CT and TCP. The low Pi response regulator PhoB was capable of shorting this regulation cascade by binding and repressing the *tcpPH* promoter (Figure 2B). This resulted in impaired colonization by a *V. cholerae* Δ*pst* strain in the infant mouse and decreased expression of CT and TCP [417]. However, under the normal high Pi conditions of the gut, the AphA/AphB regulatory cascade would serve to promoter TCP and CT production.

cyclic-di-GMP: A Pi/PhoB messenger of motility and biofilm formation

During the last decade, several investigations point-out the implication of c-di-GMP in various bacterial phenotypes and fundamental processes including Pi homeostasis and bacterial virulence. c-di-GMP [bis-(3′-5′)-cyclic dimeric guanosine monophosphate] is a ubiquitous soluble molecule and a bacterial second messenger. c-di-GMP induces biofilm formation by stimulating the biosynthesis of various adhesins and exopolysaccharides and inhibits bacterial mobility by controlling the switch between planktonic and sedentary lifestyles (For a review see refs [424,425]). The level of c-di-GMP is enzymatically and spatio-temporally controlled in bacterial cells by the GGDEF and the EAL domains of many diguanylate cyclases (DGC) and phosphodiesterases (PDE) (For a review see [426]).

The link between Pi / Pho and bacterial virulence via c-di-GMP signaling has been investigated in *V. cholerae.* Twenty-two *V. cholerae* genes have been predicted to encode an EAL domain protein including VieA [427]. It has been shown that expression of the PDE VieA positively regulates virulence gene expression[428], while ectopic expression of a DGC reduced the induction of virulence genes during infection using the infant mouse model of cholera[429]. VieA also positively regulates motility and flagellar synthesis genes[430], and

negatively regulates *vps* expression. These findings suggest that c-di-GMP assists in the transition from environment to host via regulation of *V. cholerae* behavior. Indeed, Tischler et al. [431] and Pratt et al. [432] have shown that the *vieA(E170A)* mutation abrogates PDE activity, and increases intracellular c-di-GMP levels leading to hypo-motility and hyper-biofilm phenotypes. Sometime after, the same team demonstrated that a Δ*pst* mutation could restore motility and decrease biofilm formation of the *vieA(E170A)* strain [433]. In the study, the authors also demonstrated that the *V. cholerae* WT strain grown in low Pi increased its motility and decreased biofilm formation (Figure 2B). This was due to degradation of [c-di-GMP] by AcgA, another EAL domain containing PDE. In this condition, PhoB indirectly activated the transcription of the *acgAB* operon that encodes two enzymes with opposing activities on the intracellular [c-di-GMP]. It seems that in low Pi, the PDE activity (AcgA) overrides the DGC activity (AcgB) [433].

The way that PhoB could actually regulate *V. cholerae* virulence remains ambiguous. Indeed, high c-di-GMP levels *in vivo* attenuated *V. cholerae* virulence. This was confirmed using recombination-based *in vivo* expression technology (RIVET) to monitor *toxT* expression [429]. However, as mentioned above, PhoB decreases c-di-GMP level while inhibiting ToxT-dependent virulence.

Even so, the link between Pi homeostasis and c-di-GMP signalling, suggests a regulatory model in which PhoB activation shuts down virulence gene expression during the late stages of infection. This also occurs when extracellular Pi becomes limiting and a concomitant induction of gene expression that promotes bacterial survival and dissemination in low Pi aquatic environments [433].

Roles of PhoB and Pi response in various virulence-related phenotypes of *Pseudomonas spp.*

Many bacterial species belonging to the genus *Pseudomonas* have been characterized, demonstrate metabolic variability, and are able to colonize a wide range of niches such as soil, water, and plant or animal tissue. The most-studied *Pseudomonas* species include the opportunistic pathogen *P. aeruginosa* and the plant growth-promoting *Pseudomonas fluorescens*.

Pi limitation switches-on harmful and lethal phenotypes in *P. aeruginosa*

P. aeruginosa is responsible for severe nosocomial infections and chronically colonizes lungs of cystic fibrosis (CF) patients leading to morbidity and mortality [434],[435]. Infection with Gram-negative pathogens, leads to Pi reduction in the plasma to a suboptimal level for bacterial growth [436], and the Pi concentration measured from sputum of CF patients ranged from 12 to 14 mM [437]. In these conditions, *P. aeruginosa* synthesizes several exo-products, including the C phospholipases [438] that are encoded by non-tandem genes in the *plcSR* operon. These PLCs are also induced by osmoprotectant compounds from eukaryotic and prokaryotic cells. They also cause human platelet aggregation, paralysis, vascular permeability, and death when injected into mice. A *P. aeruginosa* strain lacking *plcR* was more hemolytic and virulent than the WT parent strain. In addition, the Δ*plcR* strain was highly lethal in the mouse burn model of infection when grown in low Pi compared to in high Pi [439]. Furthermore, in a high osmotic environment, the induction of PLC-N, but not PLC-H, requires limiting Pi and PhoB [440]. This indicates a relevant pathogenic potential of PLC in the hyperosmotic conditions of the lungs of cystic fibrosis patients. Furthermore, PLC is also known to degrade phosphatidylcholine, a component of lung surfactant that may provide essential Pi nutrients with the potential to enhance lung colonization and cause atelectasis.

It was reported that Pi-starvation of *P. aeruginosa* in the distal gut coupled with the administration of analgesic opioids led to PhoB induction of the expression of *phzA1/2* (phenazine biosynthesis), *rhlR,* and *lasR* (which encode response regulators of the quorum sensing systems Rhl and Las). This was directly linked to *P. aeruginosa* enhanced virulence (pyocyanin toxin production) and drug resistance during development of human sepsis [441-443]. Increased pyocyanin toxin production was PhoB-dependent via RhlR and PhzA1/2 when Pi was limited. However, in Pi-sufficient conditions, RhlR stimulated pyocyanin production in a PhoB-independent way through PQS (*Pseudomonas* Quinolone Signal) and/or low iron availability [441]. By contrast, the host supplemented with excess Pi was less susceptible to *P. aeruginosa* infection [444] . The excess of Pi attenuated bacterial virulence by decreasing opioid-induced pyocyanin production.

Highly virulent multi-drug-resistant clinical strains of *P. aeruginosa* showed an over-production of PstS and the ability to kill 60% of surgically injured mice[445]. This virulence

phenotype led to a PstS-rich structure induced by PhoB in low P_i that allows scavenging of Pi within the host while remaining less accessible to the immune system. In contrast, the Δ*pst* strain was harmless and did not produce PstS [445,446]. Furthermore, the rough cells (producing PstS-rich appendages at cell surfaces) formed less biofilm than smooth cells [446]. *P. aeruginosa* could also cause red death in 60% of *Caenorhabditis elegans* nematodes [447]. This lethal phenotype appears to be activated by a triangulated response to low Pi between PhoB, MvfR-PQS quorum sensing, and the pyoverdin iron acquisition system.

All these reports emphasize the central role of Pi in switching on the virulence of *P. aeruginosa.* They also suggest that this opportunistic pathogen can use Pi deficiency as an environmental signal of host injury and then shift to virulent and lethal phenotypes.

Pi stress changes the motility and biofilm formation of *Pseudomonas spp.*

The studies on motility and biofilm formation phenotypes of *Pseudomonas spp* due to Pi stress reported changes similar to those occurring in *V. cholerae*. In *P. aeruginosa*, *Pi* limitation promoted bacterial hyper-swarming through PhoB up-regulation of *rhlR* expression resulting in increased rhamnolipid production [448]. The rhamnolipid biosurfactants facilitate swarming motility. This may explain the hyper-motile phenotype exhibited by the Δ*pst* mutant and the motility defect observed in the Δ*phoB* mutant [449].

Moreover, Monds et al. [450] showed that *P. fluorescens* lost its ability to form biofilm in response to Pi limitation through Pho regulon activation. For biofilm formation, *P. fluorescens* requires the production of LapA adhesin that is responsible for the transition from reversible to irreversible attachment, and that is stimulated by c-di-GMP. In low Pi conditions, the level of c-di-GMP was decreased through PhoB inducing the PDE activity of RapA. This inhibition of *P. fluorescens* biofilm formation in low Pi also requires the Pho-dependent activation of ApaH, an enzyme that degrades Ap4A, the dinucleoside polyphosphates suggested to be an "alarmone" that signals the onset of oxidative stress [451].

Effect of phosphate availability on virulence gene expression in pathogenic *E. coli*

We previously reviewed the importance of the Pho regulon for *Escherichia coli* virulence and the relationship between the Pi metabolism and pathogenicity [338,452]. More recently,

studies have been reported virulence mechanisms depending on the environmental Pi or the Pho / Pst systems.

Extra-intestinal pathogenic *E. coli* (ExPEC)

ExPEC are an important group of pathogenic *E. coli* that cause a wide range of human and animal diseases. The constitutive expression of the Pho regulon through inactivation of the Pst system was demonstrated in ExPEC strain 5131 causing porcine septicemia [453,454], the avian pathogenic *E. coli* (APEC) serogroup O78 strain χ7122 [455,456]*, and* uropathogenic *E. coli* (UPEC) strain CFT073 [457]. In all these strains, the Pho constitutive expression resulting from deletion of the Pst system attenuated virulence and virulence attributes, including sensitivity to hydrogen peroxide and serum, decreased production of type 1 fimbriae and cell surface modification. In the case of UPEC strain CFT073, the constitutive activation of the Pho regulon altered expression of regulators *fimB*, *ipuA* and *ipbA* (encoding recombinases, mediating inversion of the *fim* promoter), and decreased the level of the alarmone ppGpp that led to reduced type 1 fimbriae production and mouse bladder colonization [457]. However, in APEC, the PhoR-mediated constitutive activation of the Pho regulon was critical for virulence, rather than inactivation of the Pst system. Indeed, a point mutation in *phoR*, which resulted in constitutive activation of the Pho regulon independently of Pi transport and to inactivation of the Pst system, resulted in *in vivo* virulence attenuation [455].

Diarrheagenic *E. coli* and *Citrobacter rodentium*

For intestinal pathogenic *E. coli*, the implication of the Pho regulon in virulence modulation was demonstrated for enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). These 2 diarrheagenic *E. coli* groups, together with *C. rodentium*, belong to a class labeled as attaching-effacing *E. coli* (AEEC). They share the locus of enterocyte effacement (LEE) encoding the type III-secretion system (T3SS) and its effectors and the ability to cause the A/E phenotype. A deletion of the Pst system in different strains among the EPEC showed either an *in vitro* or *ex vivo* defect of the attachment and adhesion to epithelial cells. Moreover, it has been shown that a *pst* mutant of *C. rodentium* is less virulent for mice, its natural host [458], and recent studies reported that the Pho regulon regulates the virulence of *C. rodentium* through PhoB control of DegP (serine protease) and NleG8 (a T3S protein) [459]. In EHEC,

PhoB directly modulated LEE gene expression as well as proteins secreted by T3SS (Chekabab et al. in press). Moreover, EHEC can release Shiga toxins (Stx) that cause bloody diarrhea and hemolytic uremic syndrome (HUS). *In vivo* Pi-limiting media seem to increase the production and secretion of Stx2 by increased *stx2* transcription mediated by PhoB (Chekabab et al. in press). Also, we previously showed that the PhoB protein is critical for EHEC persistence in co-culture with amoebae [460]. These could be linked to EHEC adaptive response to low Pi after exit from the mammalian host, as EHEC has been found in aquatic poor nutrient environments [461], where it might co-exist with protozoan predators that would promote its survival outside of the mammalian gut.

Other phenotypes and bacterial virulence tied to phosphate and the Pho regulon

In recent years, additional reports have linked extracellular Pi availability, the PhoB regulator and/or the Pst system to different bacterial phenotypes and virulence-related determinants. Here we describe some virulence phenotypes where the genetic mechanisms are not yet fully elucidated.

Biofilm formation and antibiotic resistance

Biofilm formation by the plant pathogen *Agrobacterium tumefaciens* is enhanced in low Pi conditions through increased attachment by unipolar polysaccharide (UPP), and is controlled by the PhoB-PhoR regulatory system that is also essential for viability of *A. tumefaciens*[462,463]. *Proteus mirabilis* is the most common aetiological agent responsible for complicated urinary tract infections (UTIs). Its survival in the urinary tract depends to its ability to produce of a battery of virulence factors, including urease, flagella, fimbriae, haemolysin and formation of crystalline biofilm on indwelling catheters[464-466]. *The a*bolition of the Pst system in *P. mirabilis* caused a defect in biofilm formation when grown in human urine[467]. As long as bacteria use the Pho regulon to regulate attachment to surfaces and aggregate to form biofilms, formation of these sessile communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections[462].

The lower susceptibility of biofilm-grown bacteria to antimicrobial agents (antibiotics, biocides) has been extensively investigated[468-470]. Vilain et al.[471] showed that Pi deprivation of *E. coli* in gel-entrapped conditions (like bacteria in natural biofilms), is associated with a high β-lactam antibiotic resistance (latamoxef). Furthermore, it is known that Pi excess in the medium suppresses the biosynthesis of many secondary metabolites such as antibiotics[472,473]. As mentioned above, cross-talk activation of PhoB comes from VanS, the HK partner in VanR-VanS TCS that orchestrates vancomycin resistance in *Enterococcus faecium*[419]. These findings imply Pi homeostasis and PhoB in a complex regulation of antibiotic stress resistance.

Oxidative stress resistance

Several studies reported the activation of oxidative stress resistance in conditions of Pi starvation or when Pst is abolished, and that both situations activate the Pho regulon. These reports concerned different bacteria species: *Sinorhizobium meliloti*, *P. aeruginosa* and *A. tumefaciens* [474], *V. cholerae* biotype El Tor [475]; avian pathogenic *E. coli* [456] and *C. rodentium* [458]. Taken together, these studies indicate that Pho regulon activation leads to increased gene expression of catalases that protect bacteria by degrading hydrogen peroxide. There was also greater gene expression of genes encoding DNA protection proteins and superoxide dismutase. However, since the major oxidative stress response regulators, OxyR and SoxRS seem not to be under control of the Pho regulon induction, the pathway linking the reduction of oxidative stress functions to Pi management remains unknown.

Bacterial secretion systems

Gram-negative bacteria use a variety of secretion systems to target proteins to both prokaryotic and eukaryotic cells. Secretion systems such as the type III, IV and VI secretion (T3SS, T4SS and T6SS) pathways, translocate substrates directly into recipient cells in a contact-dependent manner, and constitute important virulence factors. Recent observations link the regulation of Pi homeostasis with Sec-independent secretion systems in some bacterial species. A well-studied example occurs in the pathogen *Edwardsiella tarda.*

E. tarda has a broad range of aquatic species in addition to fish[476]. It can also be found in the intestinal tract of birds, reptiles, and mammals including humans[477]. The T3SS

and T6SS were determined as the two most important virulence mechanisms in *E. tarda*[478,479]. In response to both, Pi and iron availabilities, Chakraborty et al.[480] investigated the effects of PhoB and Fur regulators on T3SS and T6SS in in this organism. The authors demonstrated that EsrC (a positive regulator of both T3SS and T6SS), and PhoB, both bind to the *evpA* promoter (*E. tarda* virulence protein) to regulate directly and positively gene transcription of T6SS. In addition, PhoB interacts with PhoU to activate *esrC*. Fur however, senses high iron and inhibits EsrC-binding to *evpA*. It was then concluded that PhoB and Fur negatively and indirectly cross-talk via unidentified factors to regulate the T3SS and T6SS in *E. tarda*. The regulation of virulence genes through PhoB/Pi pathway becomes more complex in interplay with other nutrient master regulators.

Pho regulon expression during infection and immune system hijack

Depletion of extracellular Pi after a surgical injury via phosphatonin induction of phosphaturia resulted in a reduction of Pi in serum [481]. Under these Pi-limiting conditions, it has been shown that in *Bacteroides fragilis* PhoB was necessary for successful infection and survival in peritoneal abscesses [482]. In this study, activation of the Pho regulon induced a shift of *B. fragilis* from gut symbiosis to pathogenicity. Recently, it has been shown that *Mycobacterium tuberculosis* requires the Pst system to negatively regulate activity of RegX3 (a PhoB analogue) in response to *in vivo* available Pi [483]. The authors showed that the *M. tuberculosis* Pst system was essential for virulence in mice and to counteract IFN-γ-dependent host immunity. Using selective capture of transcribed sequences (SCOTS), we previously showed that APEC O78 strain χ7122 can express PhoB *in vivo* in an infectious context, in infected chickens [484]. However, the *pst* deletion increases susceptibility of APEC O78 to rabbit serum, while this strain was not killed by chicken serum [485]. This suggests the presence of species specific differences in host innate immune defenses and complement-mediated killing.

Summary

All bacterial species including pathogens and commensals are equipped to cope and adapt to nutritional limitation. In most cases, the same input signal such as Pi availability is directly controlled by Pst and the PhoB/R TCS, allowing a Pi homeostasis response. Depending on the

level of Pi signal, pathogenic bacteria regulate different downstream systems such as quorum sensing regulatory networks and production of c-di-GMP, adhesins, or cytotoxins. This results in virulence variation and different adaptive changes including biofilm formation, motility, and antimicrobial and oxidative stress resistance.

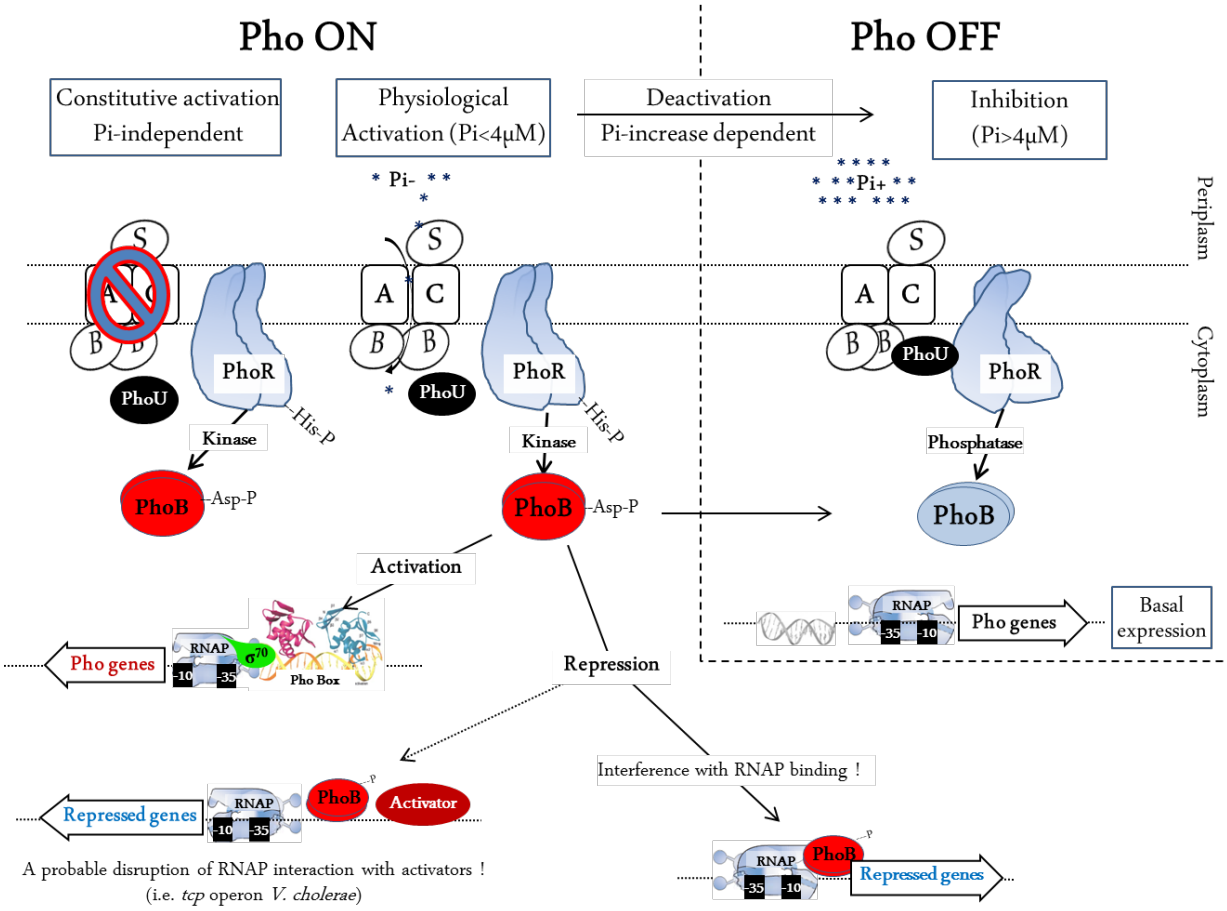

Figure 1. Model for transcription regulation by PhoB and transmembrane signal transduction by environmental Pi and the Pst system in *E. coli*.

Two processes are involved in Pho regulon control: inhibition when Pi is in excess and activation under Pi limitation. The Pst transport system is associated with the PhoR histidine kinase/phosphatase, which controls the phosphorylation state of the response regulator PhoB.
At high Pi concentrations (> 4μM), PhoR might interact with the PstSCAB-PhoU complex, which represses its autophosphorylation and acts as a PhoB phosphatase.
In contrast, low extracellular Pi concentrations sensed by Pst system, which increases the kinase activity of PhoR, resulting in the accumulation of phosphorylated PhoB~P. Likewise, loss-of-function mutations in the *pst* genes lead to the constitutive phosphorylation of PhoB, regardless of the external Pi concentration.
The Pi binding protein PstS acts as the primary sensor of external Pi concentration. When the Pi concentration is low (< 4μM), PhoR undergoes conformational change, resulting in its release from the repressor complex and autophosphorylation. PhoR is the histidine kinase/phosphatase that donates a phosphoryl group to PhoB when environmental Pi is limiting and removes the phosphoryl group from PhoB~P when environmental Pi is abundant. PhoB is a typical winged-helix response regulator that upon aspartyl phosphorylation forms a dimer, which binds to DNA sequences upstream of Pho regulon genes to recruit RNA polymerase (RNAP) and initiate transcription. In some cases the transcription is repressed by probable disruption of RNAP interaction with other activators.

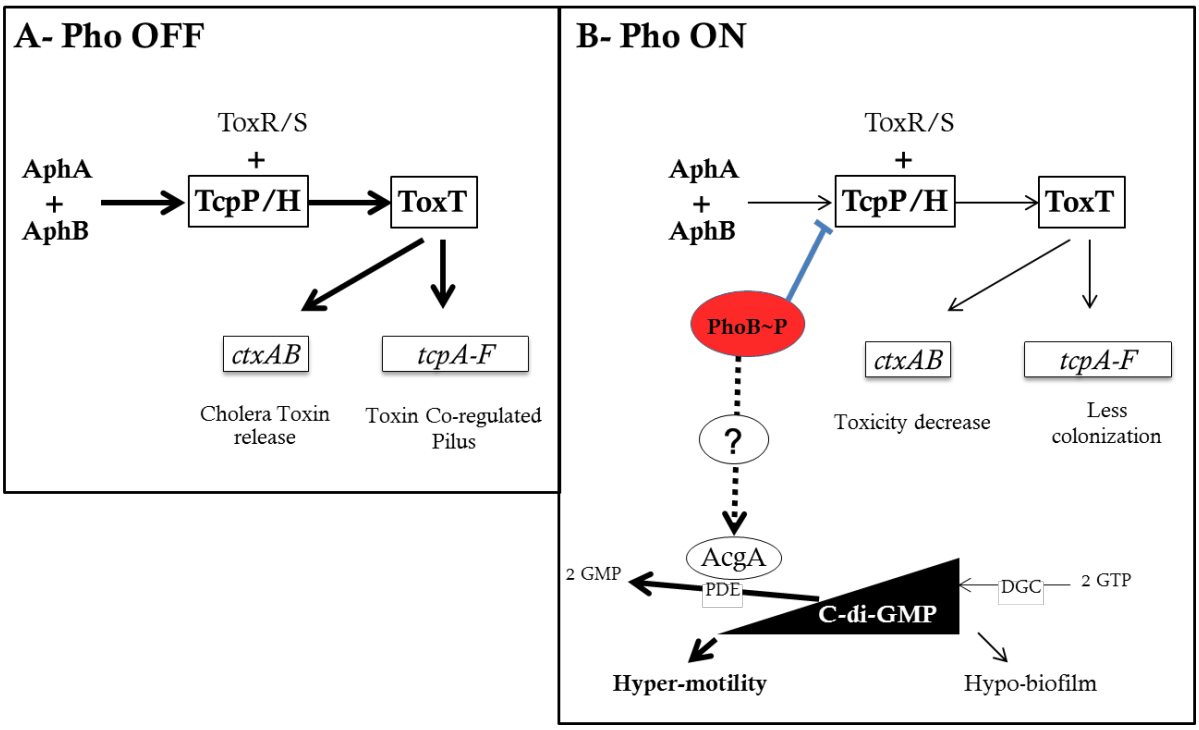

Figure 2. The *V. cholerae* virulence cascade and the mechanisms of Pi/Pho regulation of virulence, motility and biofilm formation.

A. *V. cholerae* virulence cascade. In high Pi conditions (Pho OFF) and in conditions favoring gene expression of AphA and AphB regulators, AphA cooperates with AphB to activate the *tcpPH* promoter. TcpPH cooperatively with ToxR/S, activates the *toxT* promoter. ToxT then activates the *tcpA-F* and *ctxAB* promoters. This increases colonization through formation of the toxin co-regulated pilus (TCP) and cytotoxicity through Cholerae toxin (CT) release.

B. In low Pi conditions (or Δ*pst* mutation), PhoB~P binds to Pho-box located between the tcpPH promoter and the binding sites of AphA and AphB. This results in *tcpPH* repression which reduces the downstream ToxT-dependent virulence cascade. C-di-GMP is synthesized from 2 GTP molecules by diguanylate cyclases (DGCs) and degraded to 2 GMP molecules by phosphodiesterases (PDEs). Studies in *V. cholerae* have shown that c-di-GMP increases biofilm formation and reduces the motility and *toxT* expression. In low Pi (or Δ*pst* mutation) PhoB induce indirectly the expression of *acgAB* which results in degradation of c-di-GMP leading to increase motility and decrease of biofilm formation.

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Chapitre II : Association et survie des EHEC avec *Acanthamoeba castellanii*

i. Article de recherche # 1. «Survie des EHEC en présence d'*Acanthamoeba castellanii* et sa dependence au régulon Pho»

Survival of enterohemorrhagic *Escherichia coli* in the presence of *Acanthamoeba castellanii* and its dependence on Pho regulon

Samuel Mohammed Chekabab, France Daigle, Steve J. Charette, Charles M. Dozois and Josée Harel (2012) MicrobiologyOpen 1(4): 427–437

Objectifs et approche utilisée

Cette étude publiée dans MicrobiologyOpen avait pour objectif de tester le potentiel des EHEC à persister au-delà de 21 jours en présence des amibes de l'espèce *A. castellanii*. Les résultats présentés montrent que la souche EHEC EDL933 est capable d'être cultivable plus longtemps en présence de l'amibe. Ce phénotype d'association, EDL933/amibe, est indépendant aux Stx. En revanche le régulateur PhoB est requis pour cette association. Par ailleurs, la présence des EHEC ralenti significativement la croissance des amibes en comparaison avec à la présence de souche *E. coli* non-pathogène ou en culture d'amibes seules.

L'étude a été réalisée en utilisant des techniques de monoculture et co-culture cellulaire, de dénombrements des amibes et des CFU bactériens, de dénombrement des bactéries VBNC (Kit Live-Dead BacLight), de microscopie électronique et à fluorescence, ainsi que le dosage du phosphate inorganique dans les milieux de culture. J'ai créé une partie des mutants EDL933 Δ*phoB* et Δ*pst* utilisés dans cette étude et le mutant Δ*stx* provient de l'étude effectuée dans le laboratoire de Dre Christine Martin à l'INRA [486].

Contribution de l'étudiant

J'ai effectué presque la totalité des expérimentations. France Daigle m'a aidé à apprendre à la manipulation des amibes dans son laboratoire et Steve Charette a effectué les observations en microscopie électronique à l'université de Laval à Québec. Aussi, j'ai écrit et soumis cet article qui a été corrigé par mes directeurs et collaborateurs.

Survival of enterohemorrhagic *Escherichia coli* in the presence of *Acanthamoeba castellanii* and its dependence on Pho regulon

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) are involved in outbreaks of food-borne illness and transmitted to humans through bovine products or water contaminated by cattle feces. Microbial interactions are one strategy used by pathogenic bacteria to survive in the environment. Among protozoa, the free-living amoebae are known to host and protect several water-borne pathogens. In this study, the interaction between EHEC and the predacious protozoa *Acanthamoeba castellanii* was investigated. Using mono- and co-cultures, growth of both organisms was estimated for three weeks by total and viable cell counts. The numbers of EHEC were significantly higher when cultured with amoebae than without, and less EHEC shifted into a viable but non-culturable state in the presence of amoebae. Using several mutants, we observed that the Pho regulon is required for EHEC growth when co-cultured with amoebae. In contrast, the Shiga toxins (Stx) were not involved in this association phenotype. Co-cultures monitored by electron microscopy revealed a loss of the regular rod shape of EHEC and the secretion of multilamellar vesicles by the amoebae, which did not contain bacteria. As the interaction between *A. castellanii* and EHEC appears beneficial for bacterial growth, this supports a potential role for protozoa in promoting the persistence of EHEC in the environment.

Keywords. EHEC*, A. castellanii,* bacterial persistence, Pho regulon, Shiga toxin.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are a group among Shiga toxin (Stx)-producing *E. coli* involved in outbreaks of food-borne illness. It is assumed that Stx2 plays a pivotal role in the development of hemolytic uremic syndrome (HUS), a possible life-threatening complication that can arise from EHEC infection. EHEC cause attaching/effacing of microvilli on enterocytes in humans, resulting in acute watery diarrhea. This is linked to the *Locus of Enterocyte Effacement* (LEE), which encodes the Eae adhesin and type III secretion system (T3SS) and some of its effectors [487,488].

EHEC are widely distributed in domestic ruminants that are considered an important route for transmission to humans [489]. Cattle are the main reservoir for EHEC, especially serotype O157:H7 and can result in zoonotic transmission through the consumption of raw or undercooked contaminated beef and other bovine products. However, fresh salad greens, fruit juices, unpasteurized milk, and water contaminated by cattle manure are other sources of human infection by EHEC. The persistence of this dangerous human pathogen in animals, animal products, plants, soil, and water is well established [490,491].

Free-living protozoa are also found in a diverse range of habitats, from water and soils to the intestinal tract of a range of vertebrate hosts [204]. They participate in nutrient and energy turnover acting as predators controlling bacterial numbers but they also sometimes serve as reservoirs for pathogenic microorganisms. Thus protozoa could contribute to transmission of the bacteria. In fact, certain bacteria have been shown to survive ingestion by protozoa [492]. Bacterial pathogens including *Vibrio*, *Legionella*, *Mycobacterium, enteropathogenic E. coli,* and the meningitis-causing *E. coli* strain K1 multiply and/or survive within protozoa [202,204,493-496].

The *Acanthamoeba* species, which are free-living amoebae, are naturally occurring hosts of several water-borne pathogens [497,498]. These protozoa are known to resist various stress conditions [499-501]. They are commonly found in water and in other habitats and may therefore co-exist in environments with pathogens such as *E. coli*. Consequently, it has been shown that increased survival of *E. coli* O157 occurs in association with the common environmental protozoan *Acanthamoeba polyphaga* [502]. Moreover, transcriptomic investigation showed that expression of Stx by *E. coli* O157 was increased during short-term

interaction with *A. castellanii* [503]. However, the study on long-term interaction on weeks of *Acanthamoeba* with *E. coli* O157 has never been addressed before.

Phosphate, an essential component of bacterial nutrition, plays an important role in cell metabolism. It is a constituent of nucleic acids, phospholipids, lipopolysaccharides, and is involved in energy transport and many catalytic processes. Bacteria meet their requirements for phosphorus through the assimilation of various phosphorus-containing compounds. Such compounds are transported into cells and then incorporated into ATP [504]. The preferable source of phosphorus in bacteria is inorganic phosphate (Pi), and under phosphate-limiting conditions, Pi is transported by the Pst (phosphate-specific transport) system while the phosphate regulon (Pho) allows bacteria to adapt to low Pi concentration but also is shown to modulate bacterial virulence [505].

Our hypothesis is that *Acanthamoeba* might be a reservoir of EHEC especially in aquatic environments. Moreover, in these environments, limited nutrient availability such as low Pi could be a nutritive stress for microorganisms. This study was designed to characterize the long-term *in vitro* association of EHEC strain EDL933 [506] with *A. castellanii*. The specific aims were to assess the involvement of the Pho regulon in these interactions and to determine the impact of Stx toxins on EHEC/amoeba interactions.

Materials and Methods

Culture of *amoebae*

In this study we used the free-living amoebae *A. castellanii* genotype T4, (American Type Culture Collection 50492) cultured as previously described [507]. Briefly, amoebae were grown without shaking in 30 ml of PYG medium [0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract, and 1.5% (w/v) glucose] in T-75 tissue culture flasks at 30°C [224].

Bacterial strains

All strains used in this study are listed in Table I. We used the EHEC O157:H7, strain EDL933 isolated from contaminated hamburger during a food outbreak in 1982 and implicated in HUS cases [506]. *E. coli* HB101, a K-12 laboratory strain, was used as negative

control. EDL933Δ*stx* (Δ*stx1,* Δ*stx2* double mutant) was graciously provided by Dr. Christine Martin (INRA).

To investigate the role of the Pho regulon, we created the isogenic mutants Δ*pstCAB* (constitutive Pho) and Δ*phoB* (inactivated Pho) in EDL933. The *pst* knockout mutant was obtained as previously described [508]. Briefly, the pKNG800K suicide vector [509] containing the Δ*pstCAB*::km construct was transferred to strain SM10λpir and was then mobilized in EDL933 by conjugation. Single-crossover integrants of strain EDL933 were selected on M9 agar containing appropriate antibiotics (ampicillin, kanamycin, streptomycin). Selection for double-crossover allele replacement was obtained by *sacB* counter-selection on LB agar plates without NaCl but containing 5% sucrose [510] and 5-bromo-4-chloro-3-indolylphosphate. To create an EDL933 *phoB* knock-out mutant, we performed the allelic exchange using the conjugative donor strain χ7213 [511]. PCR fragments; 768-bp upstream and 453-pb downstream of *phoB* gene were amplified from strain EDL933 using respectively the primers set AscI-PhoB-F (5' ggcgcgccgtggcgatgatgggcagagg 3') / phoB-H1P1-R (5'ggtcgacggatccccggaatgatttgccctgttgtaataa3') and phoB-H2P2-F (5'cgaagcagctccagcctacaagccgcgagcagctgttaaa3') / SacI-PhoB-R (5'gagctcgcggggtcatactgcgatcc3') and then ligated, using overlap PCR, to the kanamycin resistance cassette from pKD13 plasmid [512] amplified using the primers set phoB-H1P1-F (5'ttattacaacagggcaaatcattccggggatccgtcgacc3') / phoB-H2P2-R / 5'tttaacagctgctcgcggcttgtaggctggagctgcttcg3'). The resulting Δ*phoB*::km construct was digested with AscI and SacI, and then inserted into the conjugative plasmid pMEG-375 cut with the same enzymes. The resulting construct was transferred to strain χ7213, which is a Δ*asd* auxotroph for diamino pimelic acid (Dap) and was then mobilized in EDL933 by conjugation. Double-crossover integrants of strain EDL933 were selected on modified LB agar containing kanamycin but without Dap. The selected mutant EDL933 derivatives were confirmed to contain a deletion in the *pst* operon or *phoB* gene, respectively as determined by PCR amplification and sequencing (Eurofins MWG Operon). They were tested for alkaline phosphatase activity that measures the activity of Pho regulon as described previously [513]. Antibiotics or supplements were used at the following final concentrations, when required:

ampicillin 50 μg mL^{-1}, kanamycin 50 μg mL^{-1}, streptomycin 100 μg mL^{-1}, Dap 12 μg mL^{-1} and 5-bromo-4-chloro-3-indolylphosphate 40 μg mL^{-1}**.**

Amoebae-bacteria association experiments

Static monocultures and co-cultures of amoebae (10^5 cells mL^{-1}) and *E. coli* (10^6 CFUs mL^{-1}) were maintained without agitation at 30°C in PYG medium 1:5 diluted in PBS [224]. Samples (1.2 mL) were taken at days 1, 5, 9, 14, and 21. Total bacteria were estimated by counting the number of colony-forming units (CFUs), with the limit of detection set at 10 CFUs. *A. castellanii* densities were estimated from the same samples by counting the number of amoebae stained with 0.4% Trypan Blue for a few minutes with a haemocytometer under an inverted microscope. To determine if amoebae provide any bacterial protection during the association experiment, a part of the samples taken at days 9, 14 and 21 were also treated with gentamicin (2 mg mL^{-1}) for 2 h prior to amoebae lyses with 0.5% SDS, and then monitored for bacteria by plating followed by CFU counts the next day.

Estimation of viable but non-culturable bacteria

Bacteria in the viable but non-culturable (VBNC) state are defined as those that fail to grow on routine bacteriological media, but are alive and demonstrate very low levels of metabolic activity [514].The numbers of viable cells were determined using a LIVE/DEAD BacLight Bacterium Viability kit (Molecular Probes, Inc.) according to the manufacturer's instructions and as previously described [515]. The counts of viable cells (green only) were made with two technical replicates of diluted samples using multi-spot fluorescence microscopy slides. The VBNC number was calculated by subtracting the cultured number (i.e. CFU) from plate counts from the total number of viable bacteria as determined by fluorescence.

Transmission electron microscopy

The interactions between the two microorganisms were observed by transmission electron microscopy (TEM). Samples (6 mL) of co-culture and each monoculture were centrifuged for 10 min at 800 x g, and were then washed in PBS. The pellets thus obtained were fixed for 1 h in 2% glutaraldehyde and 0.3% osmium tetroxide prepared in 0.1 M sodium cacodylate buffer pH 7.3. The samples were centrifuged, dehydrated and embedded in Epoxy resin prior to

being cut into ultra-thin section then stained with uranyl acetate and lead citrate. The stained sections were examined with a JEOL, JEM-1230 TEM (Tokyo, Japan) operated at 80 kV.

Determination of phosphorus level

To evaluate the effect of phosphate availability on amoeba when mono- and co-cultured with *E. coli*, we measured the total phosphorus and the total Pi remaining in the media, at days 14 and 21 of culture. To do this, we used 4 replicates of the 30 mL amoeba mono or co-cultures with either EDL933 WT or Δ*pst* strains. The supernatants were filtered, adjusted to pH 2.0 then pooled to obtain 100 mL minimal volume required for phosphate measurement by a semi-automated colorimetry method. This was done at the Centre d'expertise en analyse environnementale du Québec (Laval, Canada) using the standard molybdate-reactive P method [516].

Statistical analysis

Statistics were done using GraphPad-Prism 5.0 Software (Inc., San Diego, CA, USA). All experiments were conducted in triplicate. Standard deviation of the means was calculated from the data of samples of each triplicate time-point. A repeated-measures 2-way ANOVA and a Bonferroni post-test were used to determine at which time point growth of protozoa differed significantly between absence and presence of different bacterial strains and also to compare bacterial CFUs in the bacteria-amoeba co-cultures**.**

Results

The presence of *A. castellanii* prolongs EHEC cultivability

The interaction of EHEC O157 strain EDL933 with *A. castellanii* amoeba was investigated by a co-culture experiment over a 3-week period. It was possible to establish a typical culture profile of bacteria in the presence or absence of amoebae (Fig. 1). The presence of *A. castellanii* prolonged *E. coli* O157 cultivability after 9 days of co-culture. Indeed, individual bacteria showed reduced CFU numbers after day 9 and reached the limit of detection at days 14 and 21 (Fig. 1). Moreover, at day 9 of co-culture and after gentamicin (Gm) treatment $10^5 \pm 4.7\ 10^4$ CFU mL^{-1} of strain EDL933 was recovered after the elimination of extracellular

bacteria. However no bacteria were recovered at days 14 or 21 after Gm treatment (data not shown). In contrast, the non-pathogenic *E. coli* K-12 strain HB101 showed a rapid decrease of CFUs after day 9, either with or without amoebae. HB101, in addition to the *rfb* rough mutation carries a *recA* mutation that could be involved in its reduced viability in stress conditions.

TEM examination of *E. coli* EDL933 monocultured or co-cultured with amoebae showed typical and regular rod-shaped bacteria at day 1 (Figs. 2A and 2D). However, at days 9 and 14 of co-cultures, EDL933 exhibits an irregular shape and loss of its initial appearance (Figs. 2B and 2C). When non-pathogenic *E. coli* K-12 was used in the co-incubation experiment, only a few bacteria were still visible at 9 days post-incubation (Figs. S1C and S1D). These remaining bacteria showed an irregular morphology and their cell contents was less uniform compared to those of cells from strain EDL933 after the same incubation period (Fig. S1E).

The Pho regulon, but not Stx, contributes to EHEC persistence when co-cultured with *Acanthamoeba*

To determine the effects of one of the major EHEC virulence factors during its interaction with amoebae, the co-culture experiments were repeated using an EHEC strain defective in Stx toxins production (EDL933Δ*stx*). The results were similar to wild-type EDL933 (EDL933WT) (compare Figs. 1 and 3A). As with the WT strain, EDL933Δ*stx* significantly persisted in the presence of amoebae compared to when grown as pure culture (Fig. 3A).

To investigate the role of the Pho regulon during interactions with amoebae, we used the Pho constitutively activated strain (EDL933Δ*pst*) and the Pho inactivated strain EDL933Δ*phoB* [404]. Similarly to EDL933WT, the CFU of the Δ*pst* mutant was significantly higher after day 9 in the presence of amoebae (Fig. 3B). On the other hand, enumeration of EDL933Δ*phoB* co-cultured with amoebae, showed a 100-fold CFU reduction compared to WT strain at days 14 and 21. In addition, the EDL933Δ*phoB* mono-culture exhibited a decreased cultivability to 10^3 CFU mL^{-1} at day 9 and then reached the limit of detection at day 14 until the end of the experiment (Fig. 3C).

Next we determined the number of viable and dead bacteria at the sampling time-points of days 9, 14 and 21 as described in the Material and Methods. These were monitored

respectively by green and red fluorescence using the LIVE/DEAD BacLight Kit. These were followed by the estimation of the number of bacteria in a VBNC state. As shown in Figure 4, the absence of amoebae significantly increased the number of EDL933WT cells in a VBNC state compared with the presence of amoebae ($p<0.05$). However, for the EDL933Δ*phoB* strain, we found no significant VBNC differences between the presence and absence of amoebae. In co-culture experiments, even the EDL933Δ*phoB* cultivability defect became observable as of day 9, there was no difference in VBNC when the EDL933Δ*phoB* strain was grown in mono- or in co-culture with amoebae. Thus, inactivation of the Pho regulon resulted in bacterial entrance into a VBNC state, regardless of the presence or absence of amoebae. In addition, there were fewer EDL933WT VBNC cells in co-culture with amoebae than EDL933Δ*phoB* VBNC in mono-culture or co-cultured with amoebae. For the other EHEC mutant strains (Δ*pst,* Δ*stx*), the VBNC results were similar to those obtained for the WT strain (data not shown).

EHEC reduces the growth rate of *A. castellanii*

Amoebae cell counts were compared between individual cultures and co-cultures with *E. coli* strains (Fig. 5). In these *in vitro* conditions, the presence of the EDL933 WT strain and all mutant derivatives (Δ*stx,* Δ*phoB* and Δ*pst*) significantly reduced the growth of the amoebae after 9 days of co-culture and this reduction was more apparent at 14 and 21 days of co-culture especially for the Pho constitutive mutant EDL933Δ*pst* ($p<0.01$/$p<0.001$) (Fig. 5). However, during co-culture with the non-pathogenic *E. coli* K-12 strain no significant differences in amoebae growth or morphology were observed.

TEM observations were made to visualize the fate of *A. castellanii* in the presence of bacteria (Figs. 6 and S1). Despite the non-significant change of the viable amoebae number at day 1 between mono- and co-culture experiments with EDL933, the TEM observations showed that some amoebae cells were damaged by *E. coli* O157 (Fig. 6B). This was concomitant with the highest number of bacteria recovered at day 1 post-incubation (Fig. 1). For instance, we were not able to observe any intracellular localization of EDL933 during the 3-week period. However, we observed that most *Acanthamoeba* trophozoites turned into mature cysts at days 9 and 14 (Fig. 6E) while a significant number of multilamellar vesicles were secreted by amoebae (Figs. 6D, 6F and S1F).

As the Pho regulon is constitutively activated in the *pst* mutant, this mutant is fitted for phosphate competition with the amoebae. The concentration of phosphate remaining in media was determined after 14 and 21 days of culture. The dosages of total phosphate in a pool of 4 replicates of amoebae mono- and co-cultures were respectively 110 mg mL^{-1} and 130 mg mL^{-1}. In the same cultures, the concentrations of total inorganic phosphate were slightly different between absence and presence of either EDL933 WT or Δ*pst* (respectively 102.6 mg mL^{-1}, 108.9 mg mL^{-1}, 113.4 mg mL^{-1} at day 14 and 103.5 mg mL^{-1}, 117 mg mL^{-1}, 113.4 mg mL^{-1} at day 21). Thus, the phosphate availability failed to explain the drastic reduction of amoebae in co-culture with the EDL933Δ*pst* strain.

Discussion

This report explores the interaction of EHEC with *A. castellanii*. EHEC was able to survive for 21 days in co-culture with *A. castellanii* while an *E. coli* K-12 strain rapidly declined during co-culture and was not detected after 21 days. Moreover, the growth of *A. castellanii* was restricted in the presence of EHEC. In addition the amoebae were damaged by EHEC. However Shiga toxins appeared to be uninvolved in this long-term interaction as the amoebae growth with *stx* mutant declined similarly to that with the wild type. Other studies have shown that the presence of Stx-encoding prophage augmented the fitness of *E. coli* in co-culture with *Tetrahymena* [388] and that Stx-positive *E. coli* killed the amoebae [517]. In contrast, another report indicated that Shiga toxin lysogenic phage conferred no advantages in *E. coli* interaction with ruminant ciliates [518]. Thus, the contribution of Shiga toxins to bacterial survival when facing a protozoan seems to be variable depending on the conditions of the challenge and the protozoan used in the co-culture assay.

The decrease of amoebae in co-culture with EHEC can be due to the toxicity of EHEC, its capacity to resist digestion and/or a better capacity of the bacterial strain to compete for nutrients during culture. It was shown by Ravva and co-authors that protozoa in dairy lagoon wastewater were capable of consuming but not eliminating EHEC by retaining them in food vacuoles [519]. Moreover this group showed that the protozoan *Vorticella microstoma* ingested EHEC without digesting them. Protozoan grazing is a major trophic pathway whereby the biomass re-enters the food web [520]. Nonetheless, protozoa do not digest all

bacteria. Those known to evade digestion will result in their increase in the environment [492]. In the case of EHEC, the association with the amoebae allows the bacteria to persist**.**

In prolonged co-culture, the EDL933Δ*pst* strain reduced the growth of *A. castellanii* even more than EHEC WT while no significant differences were found for phosphate concentrations between the presence of EHEC WT and Δ*pst* in the prolonged co-culture. Thus the mechanism underling the adverse effect on amoebae growth remains unknown. Similarly, *Pseudomonas aeruginosa* was also found to suppress the growth of *A. castellanii* [521]. In this condition, it has been suggested that dependent on functional RhlR/LasR quorum-sensing systems *P. aeruginosa* formed microcolonies and biofilms that exhibited acute cytotoxicity against the protozoa [522]. In the case of EHEC especially EDL933Δ*pst* strain further investigations are needed to examine if such phenomenon is involved in the interaction with the amoebae.

Interestingly, some studies have shown a relationship between the Pho regulon and biofilm formation [338]. It is also possible that the *pst* mutant may be more adapted to compete with amoebae in an environment that are limited in nutrients [338]. This is supported by the observed increase of expression of some Pho regulon genes (*phoA*, *pstA* and *pstB*) of EDL933 when facing *A. castellanii* [503]. The Pst system is involved in phosphate acquisition as well as the molecular mechanisms that lead to turning off the Pho regulon. The Pho regulon is not only a global regulatory circuit involved in bacterial phosphate management but it could also alter other cellular responses and virulence traits that could affect the bacterial survival in association with amoebae [338,404]. However, it was observed that the virulence of the *pst* mutant from other *E. coli* pathotypes such as extraintestinal pathogenic *E. coli* (ExPEC) was attenuated [455,505] demonstrating the great complexity related to the study of bacteria-protozoa interactions.

In adverse environmental conditions, the bacterial cells can lose cultivability but remain viable. In these situations bacteria reduce general metabolic activity and enter into a VBNC state [514]. Interestingly, the presence of amoebae reduces the number of EHEC cells in the VBNC state, suggesting that co-culture with amoebae enhance EHEC fitness and persistence during prolonged periods. This could result from amoebae metabolites secretion or nutrients released from amoebae dead cells that could be useful for the surviving EHEC.

Furthermore, our results suggest that increased presence of metabolically active and culturable bacteria in association with amoebae requires PhoB activity, because the EDL933Δ*phoB* strain exhibits similarly high number of VBNC whether cultured in the presence or absence of amoeba. Similarly, it has been demonstrated that *Acanthamoeba* promotes survival of *L. pneumophila* after disinfection and resuscitate cells from VBNC [523].

Our study shows that EHEC association with amoebae allows bacteria to persist and for the first time, we observed multilamellar vesicles secreted by *Acanthamoeba* in co-culture with *E. coli*. These multilamellar structures resemble those produced by *D. discoideum*, another predacious amoeba, when incubated with comestible bacteria. These multilamellar vesicles are thought to be the accumulation of undigested products [524,525]. For *A. castellanii*, these structures were observed when the protozoan was incubated in the presence of *L. pneumophila*. In this particular case, bacteria were found packaged in these multilamellar vesicles [526,527]. The packaging of *L. pneumophila* in multilamellar vesicles is known to protect the bacteria from harsh conditions and increase their viability [526-528]. In the case of EHEC, no bacteria were seen in these structures by TEM. Such structures could contribute to a protective microenvironment and/or supplemental nutrients allowing a survival advantage to EHEC during prolonged growth conditions. Altogether, our results support the probable association between EHEC and amoebae in their natural ecosystem such as water, soil, feces, and cattle. This is in line with the previous work showing the role of *Acanthamoebae* species as host for *Vibrio cholerae* and *Vibrio mimicus* found in the aquatic environments of cholera endemic areas [228,498].Indeed, *V. cholera* and EHEC share similarities being waterborne and causing diarrhoeal disease and generally believed to be extracellular bacteria. However, in relation to *A. castellanii*, *V. cholerae* behaved as a facultative intracellular bacterium and, under the experimental conditions used, apparently established a symbiotic relationship with the amoebae [246]. In our study, although the interaction with the *A. castellanii* is beneficial to EHEC growth, we do not observe a similar behaviour by electron microscopy.

This study describes one possible lifestyle of the EHEC and may contribute to understanding its ecology that may lead to potential strategies to fight against their transmission to humans and/or the recontamination of ruminants. We demonstrated that a free-living protozoan *A. castellanii* contributes to the long-term persistence of EHEC. Considering

the small number of EHEC required for an infectious dose, the role of protozoa in food and water contamination warrants greater focus in prevention research.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

We are grateful to Christine Martin (Institut National de Recherche Agronomique, France) for her insightful comments. We thank Judith Kashul and Claudia Syed for editing of the manuscript and Frederic Douesnard-Malo and Richard Janvier for their technical help with amoebae and TEM. We also gratefully acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada to J. H. (RGPIN–25120) and CMD (RGPIN-250129-07) and a studentship to M.S.C. from Fonds CRIP (FQRNT Regroupements stratégiques 111946). SJC is a research scholar of the Fonds de recherche du Québec – Santé (FRQS).

Tables and figures

Table I. *E. coli* strains and plasmid used in this study

Strain or plasmid	***Description and relevant characteristics*** [a]	***Source or reference***
EHEC strains		
EDL933WT	*E. coli* O157:H7; Wild-Type	ATCC700927 [529]
EDL933Δ*stx*	EDL933;*Δstx1/Δstx2;*Stx-negative	[486]
EDL933Δ*pst*	EDL933; *pstCAB::Km*; Pho-regulon constitutive	This study
EDL933Δ*phoB*	EDL933*; phoB::Km*; Pho-regulon negative	This study
E. coli laboratory strains		
K-12 HB101		
	F^- *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 galK2 xyl-5 mtl-1 rpsL20*(Sm^R) *glnV44* λ^- *lacY*	Laboratory stock
SM10λpir	*thi-1 leu tonA lacY supE recA :: RP4-2-Tc::Mu λpir,* Km^r	Laboratory stock
χ7213	SM10λpir *ΔasdA4,* Km^r	[511]
Plasmid		
pKNG101	Suicide vector, *sacB* Sm^r	[510]
pKNG800K	*pKNG101, pstCAB:: km sacB* ,Sm^rKm^r	[508]
pKD13	Template plasmid, *Km*^{r}gene flanked by FRT sites. *Ap*^{r}*, Tet*^{r}	*AY048744*[512]
pMEG-375	*sacRB mobRP4 oriR6K* Cm^r, Ap^r	S. Tinge, Megan Health Inc

[a] Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Sm^r, streptomycin resistant; Tet^r, tetracycline resistant, Ap^r; ampicillin resistant.

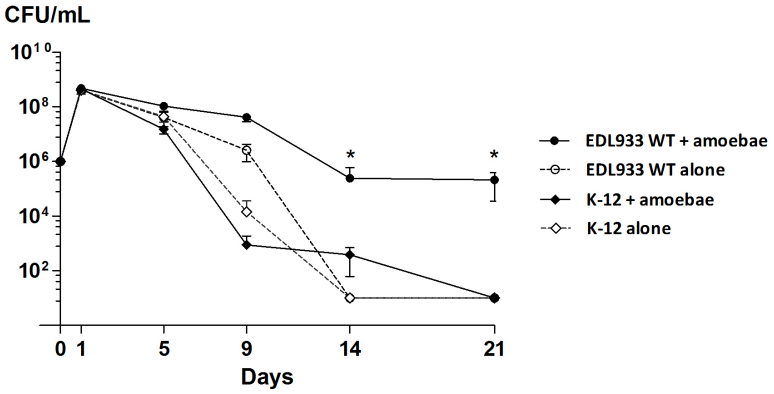

Figure 1. Prolonged cultivability of EDL933 in association with amoebae

E. coli EDL933WT (circle symbol) and *E. coli* laboratory strain K-12 HB101 (diamond symbol) were incubated with (solid lines) or without (dotted lines) *A. castellanii* for 3 weeks. The cultivability of EDL933WT maintained and prolonged to days 14 and 21 in co-culture with amoebae than without (*p<0.05). Data indicates mean ± SD of three independent experiments.

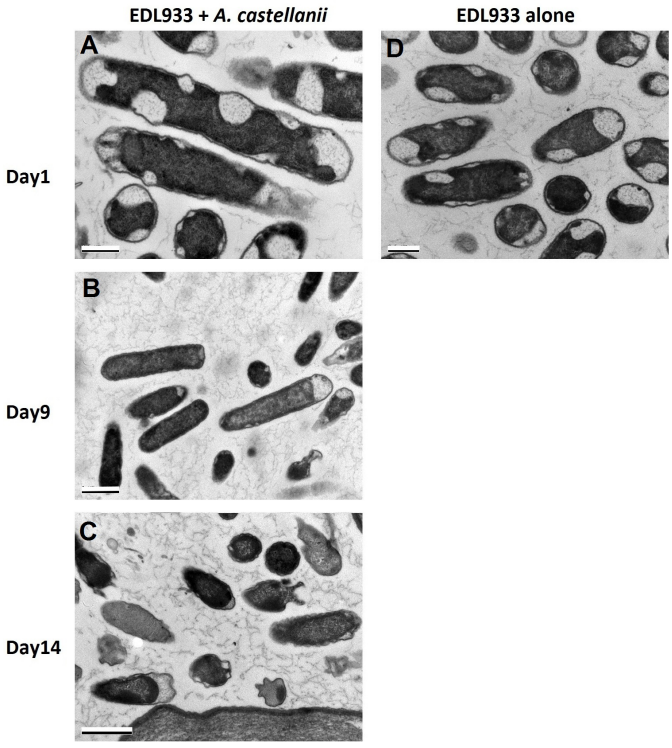

Figure 2. EDL933 morphological change in co-culture with amoebae

Electron microscope analysis of EDL933WT in co-culture with amoebae at days 1, 9 and 14 (respectively A, B and C) and after 1-day monoculture (D). Scale bars: A and D= 1 μm, B and C= 0.5 μm.

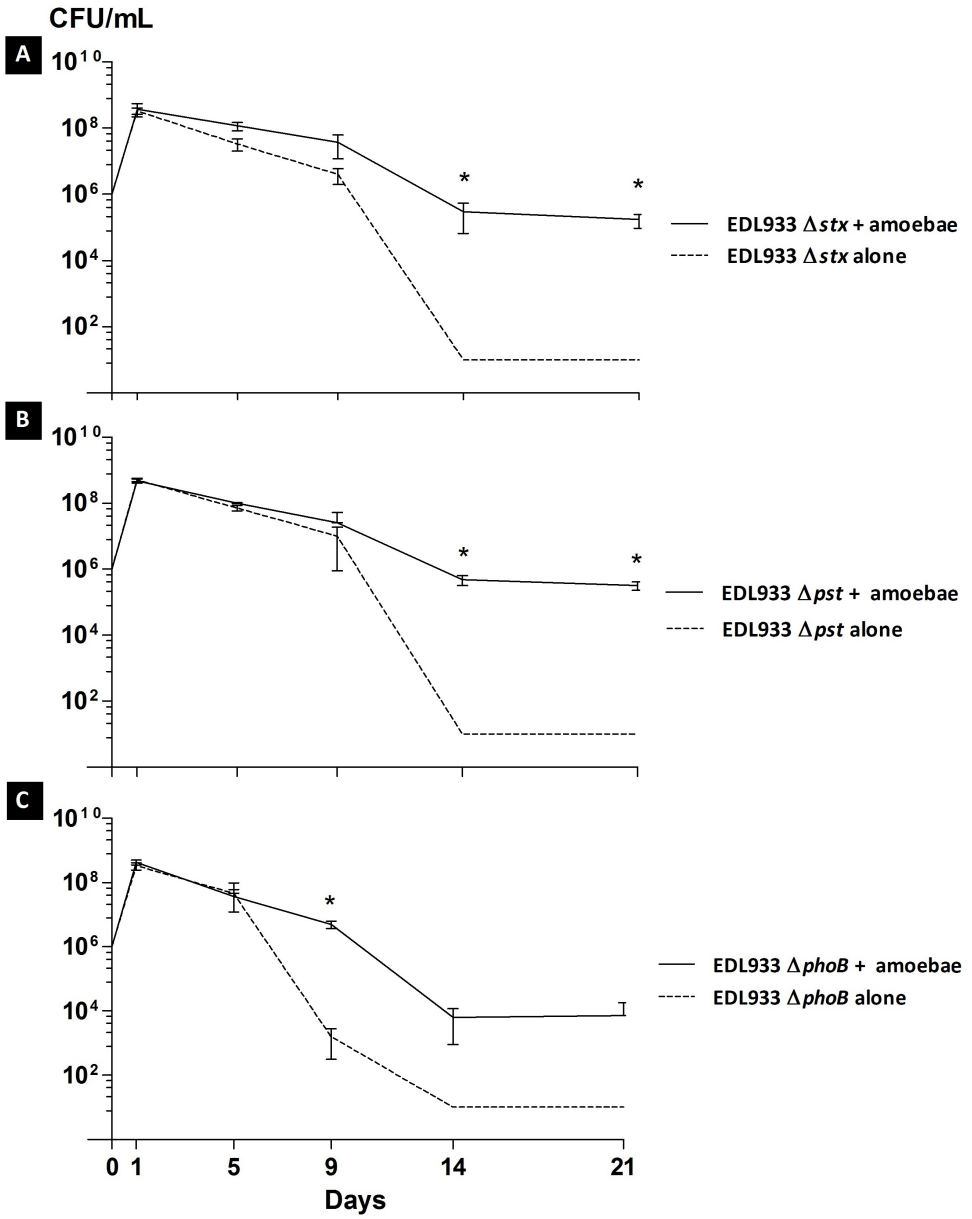

Figure 3. Δ*stx and* Δ*pst* EDL933 mutants are maintained in association with amoebae

E. coli EDL933 mutants incubated with (solid lines) or without (dotted lines) *A. castellanii* for 3 weeks. (A. EDL933Δ*stx*; B. EDL933Δ*pst*; and C. EDL933Δ*phoB*). Significant differences were found at days 14 and 21 for Δ*stx* and Δ*pst.* The strain EDL933Δ*phoB* mutant associated with amoebae showed statistical difference only at day 9. (*p<0.05). Data indicate the mean ± SD of three independent experiments.

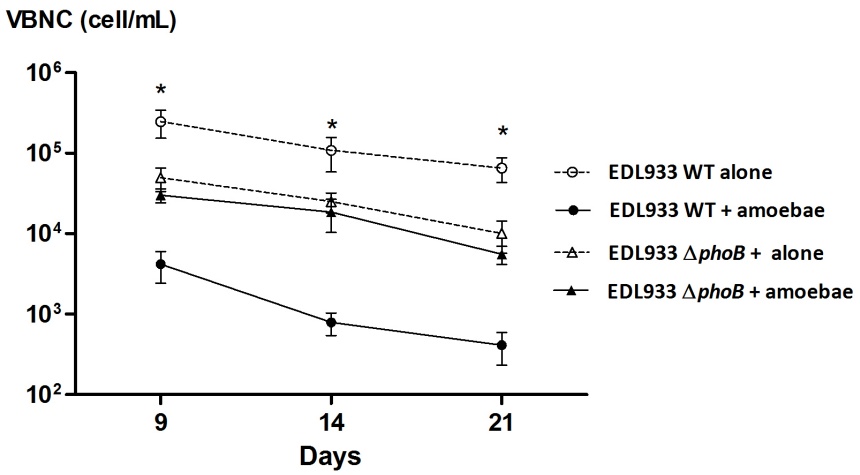

Figure 4. Viable but non-culturable (VBNC) state of *E. coli* EDL933

Calculations of VBNC of EDL933WT (circle symbol) and its mutants EDL933Δ*phoB* (triangle symbol) during 3 weeks in co-culture with *A. castellanii* (solid lines) or without (dotted lines). Samples were taken at days 9, 14 and 21. The number of the WT VBNC in mono-cultures was significantly increased compared to co-culture with amoebae (*= $p<0.05$). In contrast, no similar significant difference was observed for the VBNC number of the Δ*phoB* strain.

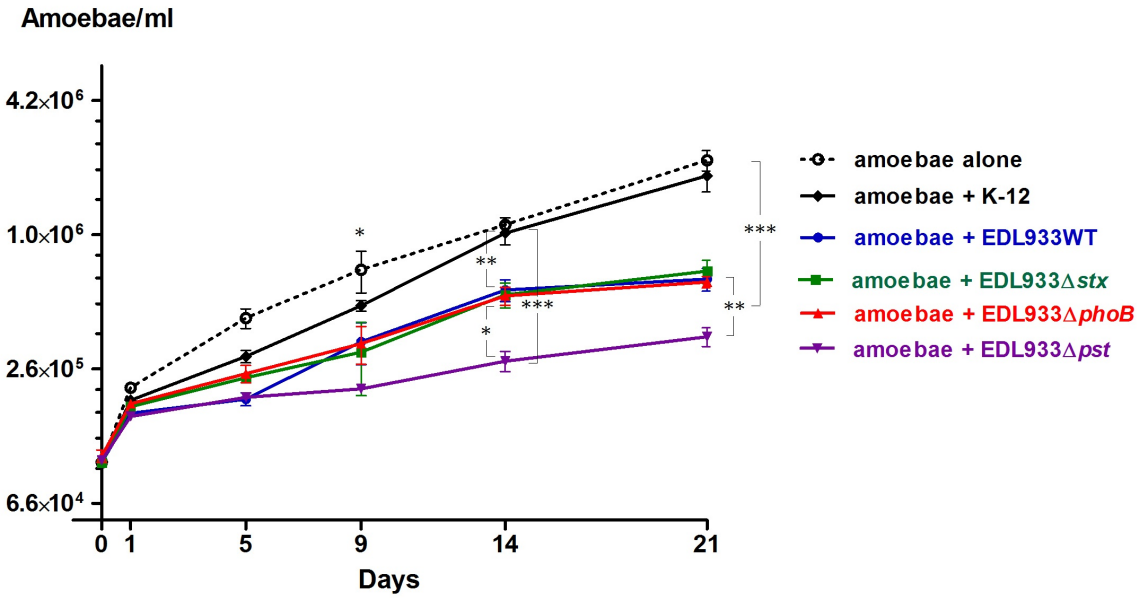

Figure 5. The growth of *A. castellanii* decreases in the presence of EDL933 and mutant derivatives

Amoebae were grown in PYG medium at 30°C for 3 weeks of static mono-culture (dotted lines) or co-cultured with *E. coli* strains (solid lines). Significant decreases in growth of amoebae in the presence of either EDL933WT or its isogenic mutants (Δ*stx,* Δ*pst* and Δ*phoB*) compared to amoebae in mono-culture or co-cultivated with *E. coli* K-12. (***$p<0.001$, **$p<0.01$, *$p<0.05$). Results are the mean ± SD of 3 independent experiments.

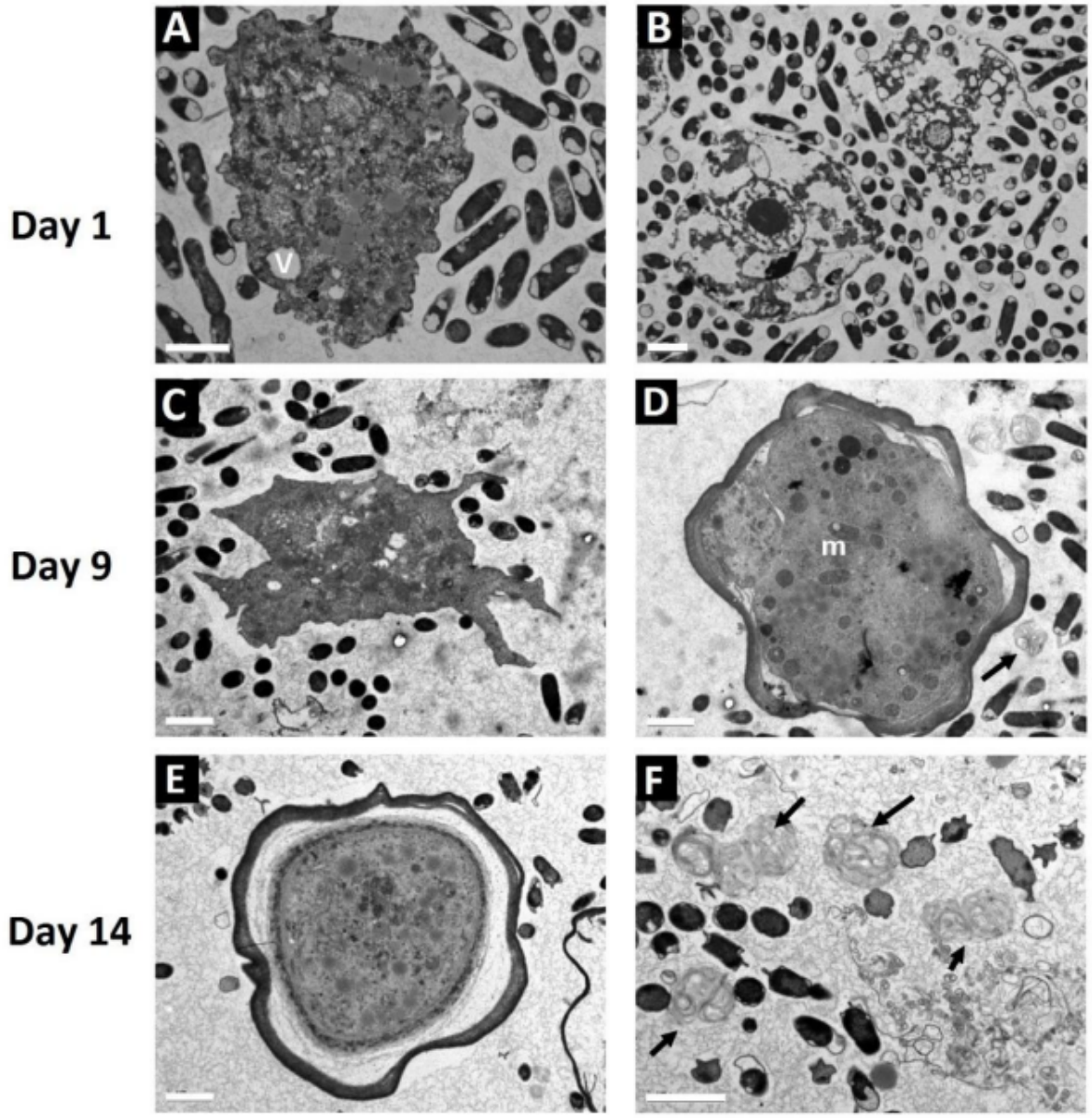

Figure 6. *A. castellanii* morphology in co-culture with EDL933

Electron micrographs showing the extracellular localisation of EDL933 and the effect on amoebae morphology. *A. castellanii* trophozoites alive (A) and killed (B) in co-culture with EDL933 at day 1 and at day 9 (C,D). A mature cyst formed by *A. castellanii* at day 14 (E). Multilamellar bodies secreted by amoebae (black arrow) at day 9 (d) and day 14 (F). M: mitochondria, V: vacuole. Scale bars = 2 µm.

Supporting information

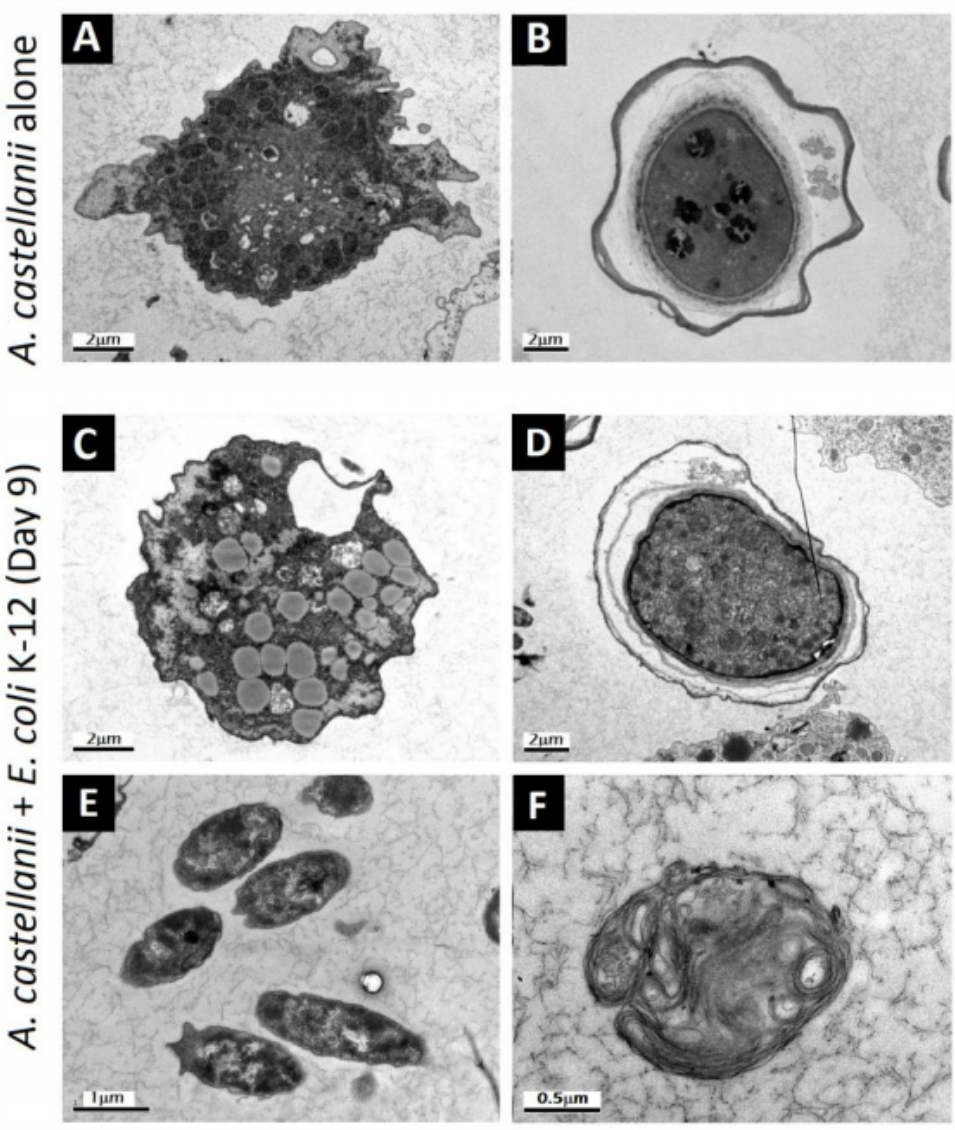

Figure S1. *A. castellanii* alone and in co-culture with *E. coli* K-12 HB101

Electron micrographs showing the 2 forms of *A. castellanii* in monoculture, the metabolic active trophozoite (A) and the dormant cyst (B) and also in co-culture with *E. coli* K-12 HB101 (C and D). Note that few *E. coli* K-12 HB101 cells are still visible at day 9 of co-culture (E) and multilamellar vesicles are also secreted in this condition (F). Scale bars: A to D= 2 µm, E= 1 µm, F= 0.5 µm.

ii. Article de recherche # 2. «Les Shiga toxines diminuent la survie des EHEC dans l'amibe *Acanthamoeba castellanii*»

Shiga-toxins decrease enterohaemorrhagic *Escherichia coli* survival within *Acanthamoeba castellanii*

Samuel Mohammed Chekabab, France Daigle, Steve J. Charette, Charles M. Dozois and Josée Harel (2013). FEMS Microbiology Letters 344(1):86-93

Objectifs et approche utilisée

Cette étude publiée dans FEMS Microbiology Letters avait pour objectif d'explorer la survie intra-amibe des EHEC et de déterminer l'implication des Stx et du SST3 dans cette survie intracellulaire. Les résultats présentés montrent qu'un petit pourcentage de bactéries, de la souche EHEC EDL933 sont capable de survivre à l'intérieur de l'amibe *A. castellanii*. Nous avons observé que ces bactéries se retrouvent dans les phagosomes à l'intérieur des amibes. La souche EDL933 ne possédant pas les gènes de Stx avait un taux de survie intracellulaire 3 fois plus important que la souche sauvage. Par ailleurs, nous avons démontré que la présence de Stx chez EDL933 à un effet cytotoxique partiel sur les amibes.

L'étude a été réalisée en utilisant des techniques de monoculture et co-culture cellulaire, de dénombrements des amibes et des CFU bactériens, de test de protection à la gentamicine, de microscopie électronique et à fluorescence, ainsi que le test de cytotoxicité des amibes par mesure de la libération de LDH.

Contribution de l'étudiant

J'ai effectué presque la totalité des expérimentations. Steve Charette a effectué les observations en microscopie électronique à l'université de Laval à Québec. J'ai aussi écrit et soumis cet article qui a été corrigé par mes directeurs et collaborateurs.

Shiga-toxins decrease enterohaemorrhagic *Escherichia coli* survival within *Acanthamoeba castellanii*

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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) are zoonotic pathogens transmitted to humans through contaminated water or bovine products. One of the strategies used by pathogenic bacteria to survive in aquatic environments is by using free-living amoebae as hosts. *Acanthamoeba castellanii* is an amoeba known to host several water-borne pathogens. This study investigates the survival of EHEC with *A. castellanii*, which could contribute to its spread and transmission to humans. We used a gentamicin protection assay as well as fluorescence and electron microscopy to monitor the intra-amoebae survival of EHEC O157:H7 over 24 hours. The results showed that EHEC were able to survive within *A. castellanii* and that this survival was reduced by Shiga-toxins (Stx) produced by EHEC. A toxic effect mediated by Stx was demonstrated by amoebae mortality and LDH release during co-culture of EHEC and amoeba. This work describes the ability of EHEC to survive within *A. castellanii* and this host-pathogen interaction is partially controlled by the Stx. Thus, this ubiquitous amoeba could represent an environmental niche for EHEC survival and transmission.

Running title. *E. coli* O157:H7 survival within amoebae

Keywords. EHEC O157:H7, *Acanthamoeba castellanii,* intracellular survival, Shiga-toxins, cytotoxicity.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) especially serotype O157:H7 is a highly pathogenic subset of Shiga-toxin producing *E. coli* (STEC), able to cause human gastrointestinal illnesses with a broad clinical spectrum, ranging from watery and bloody diarrhoea to haemorrhagic colitis [266,267]. In rare cases, disease symptoms result in the haemolytic uremic syndrome (HUS), and long-term consequences including brain damage and chronic kidney failure. The main virulence factors of EHEC are the Shiga-toxins (Stx), commonly associated with haemorrhagic colitis and HUS [141,530] and a type III secretion system (T3SS), through which the pathogen translocates effector proteins into host cells, causing attaching and effacing (A/E) lesions [531].

In the U. S. A., according to the FoodNet surveillance, over 31 pathogens are known to cause food-borne illness. Five bacterial enteric pathogens cause ≈90 % of the illness and hospitalizations each year especially among children <5 years old, and *E. coli* O157 is implicated in 3 % of the cases [532]. Human infections often occur through consumption of contaminated food products derived from cattle that are asymptomatic carriers and the primary reservoir for *E. coli* O157:H7 [280,489]. According to a CDC report [533], 69 % and 18 % of *E. coli* O157:H7 infections were of food and waterborne origin respectively. Interestingly, fresh green salad, fruits, and vegetables are becoming better recognized as important sources of human infections by *E. coli* O157:H7. In the U. S. A., EHEC O157:H7 transmission through leafy green vegetables increased from 11% to 41% between 1998 and 2007 and this was associated with contaminated seeds, irrigation water, and flooding combined with the persistence of *E. coli* O157:H7 in the environment for long periods of time (e.g., > 1 year in compost) [282].

E. coli O157:H7 is found in natural environments including soil, manure and irrigation water or contaminated seeds [305,306]. Moreover, environmental EHEC O157:H7 can live with free-living protozoa that are widely distributed in soil, sewage, water ecosystems and in the intestinal tract of a range of vertebrate hosts [204]. Free-living protozoa can resist various stress conditions and participate in nutrient and energy turnover by acting as predators controlling bacterial numbers [500,501,534]. However, sometimes protozoa also serve as reservoirs for pathogenic microorganisms. In fact, various bacteria have been shown to survive

ingestion by protozoa [246]. Bacterial pathogens including *Vibrio*, *Legionella*, *Mycobacteria,* enteropathogenic *E. coli,* and meningitis-causing *E. coli* (*E. coli* K1) multiply and/or survive within protozoa [204,212,220,267,379,492,535].

E. coli O157 and non-O157 had been shown to survive in co-habitation with the environmental protozoa *A. polyphaga* and recently with *A. castellanii* [220,380,502,536]. In addition to enhanced environmental survival, bacterial co-habitation with protozoa could induce adaptive changes in bacteria [220]. We previously observed that the co-culture of *E. coli* O157:H7 with *A. castellanii* allowed bacterial persistence over 3 weeks [536].

To extend such studies, the present report investigated the contribution of Stx and T3SS during the interaction of EHEC and *A. castellanii*. We show that EHEC survives within amoebae during a short-term co-culture. Using a gentamicin protection assay and microscopic observations, we demonstrated the involvement of the Stx but not the T3SS in the transient intracellular survival of EHEC O157:H7.

Materials and methods

Culture of *amoebae* and viability assay

The free living amoebae *A. castellanii* genotype T4, (American Type Culture Collection 50492) was cultured as previously described [507]. Briefly, amoebae were grown without shaking in 30 mL of PYG medium diluted 5 times in phosphate buffer [0.75 % (w/v) proteose peptone, 0.75 % (w/v) yeast extract, and 1.5 % (w/v) glucose] in T-75 tissue culture flasks (Sarstedt) at 30°C [537]. *A. castellanii* cells were removed from flasks, washed with PBS, and counted with a hemocytometer. Static co-cultures of amoebae (10^5 cells mL^{-1}) and *E. coli* (10^6 CFUs mL^{-1}) were maintained in T-75 tissue culture flasks without agitation at 30°C in PYG medium 1:5 diluted in PBS. *A. castellanii* viability was determined by trypan blue staining, at 1.5 and 24h post-incubation. *A. castellanii* cells were resuspended with a 5 ml pipette. Amoebae were stained with 0.4% Trypan Blue for a few minutes then the densities of amoebae either alive (white) or dead (blue/dark) were counted with a haemocytometer under an inverted microscope. The initial viability was always greater than 98% (data not shown).

Bacterial strains

EHEC O157:H7 strain EDL933 (ATCC 700927), originally isolated from contaminated hamburger during a food outbreak in 1982 and implicated in HUS cases [506], was the reference strain for these studies. *E. coli* HB101, a K-12 laboratory strain, was included in this study as a negative control. EDL933Δ*stx* (Δ*stx1*, Δ*stx2* double mutant) is described elsewhere [486,536]. EDL933Δ*escN* (non-functional T3SS mutant) [124] was graciously provided by Dr. Samantha Gruenheid (McGill University). The EDL933 wild-type (WT) and EDL933Δ*stx* green fluorescent protein (GFP)-producing strains were obtained by electroporation of a high-copy plasmid constitutively expressing GFP (pKen2) [538].

Intracellular bacterial viability

E. coli intracellular survival assays were adapted from [535]. Briefly, similarly to the association assays, *E. coli* (10^6 cells mL^{-1}) and amoebae (10^5 cells mL^{-1}) were incubated together in a one mL volume for 1.5 h in 24-well plates. The wells were then gently washed with PBS before killing extracellular bacteria by the addition of gentamicin (Gm) (150 μg mL^{-1}) for 1 hour followed by two washes with PBS. Immediately (1.5 h intracellular survival) or 24 h after Gm treatment, the amoebae were counted then lysed with 0.5% SDS and finally intracellular *E. coli* were enumerated on LB-agar plates. Here, the multiplicity of infection (MOI) was calculated as 10:1 (bacteria: amoeba). Since *in vitro* culture and grazing on the bacteria decreased cell-adherence of amoeba and led to loss of amoeba cells during washes, the bacterial survival index was calculated as the ratio of bacteria to amoeba: (Number of recovered *E. coli* (cfu) / Number of amoebae x MOI factor) X 100 [535].

Fluorescence and transmission electron microscopy

EDL933 WT and Δ*stx* strains constitutively expressing GFP were used for the qualitative intracellular assays. This was done following the same internalization experiment mentioned above, then samples were mounted and cover slipped on slides and observed at 1000-x magnification under a confocal microscope (Olympus FV1000 IX81). GFP was excited at 488 nm and detected using 520 nm filters. The images were processed using Fluoview software (Olympus).

Interactions between amoebae and EHEC were observed by transmission electron microscopy (TEM). Samples (6 mL) from the *in vitro* mono- or co-cultures were centrifuged for 10 min at 800 x *g*, and were then washed in PBS. The pellets thus obtained were fixed for 1 hour in 2% glutaraldehyde and 0.3% osmium tetroxide prepared in 0.1 M sodium cacodylate buffer pH 7.3. The samples were centrifuged, dehydrated and embedded in Epoxy resin prior to ultra-thin sectioning then stained with uranyl acetate and lead citrate. The stained sections were examined with a JEOL, JEM-1230 TEM (Tokyo, Japan) operated at 80 kV.

Amoebae cytotoxicity assay

E. coli cytotoxicity on amoebae was assessed after 1.5 h and 24 h of co-cultures in 96-well plates, by measuring the amount of cytoplasmic lactate dehydrogenase (LDH) released using the commercial assay Cytotox96 (Promega, Southampton, Hampshire, UK). According to the manufacturer's instructions, the percentage of cytotoxicity was expressed as follows = ((experimental – effectors – spontaneous) / (maximum – spontaneous)) X 100 in which the spontaneous and effectors were the amount of release of LDH of activity in the supernatant of amoebae and bacteria monocultures respectively. The maximum release was measured after cell lysis using a lysis buffer provided by the manufacturer.

Statistical analysis

Statistics were done using GraphPad-Prism 5.0 Software (Inc., San Diego, CA, USA). All quantitative results were from 3 independent experiments, each performed in triplicate. Standard deviations of the means were calculated from the data of the 3 independent replicates. We used the non-parametric ANOVA distribution with Bonferroni post-test to compare amoebae counts in the *in vitro* association assay and the percentages of bacterial survival in the Gm protection assay as well as the percentages of LDH release in the cytotoxicity assay.

Results

Absence of Stx increases EHEC survival within *A. castellanii*

To assess quantitatively the EHEC survival within *A. castellanii*, Gm protection assays were performed 1.5 h after mixing *E. coli* and amoebae using a MOI of 10:1 (bacteria: amoeba).

Immediately or 24 h after Gm-treatment, amoebae were lysed to release intracellular bacteria, and the percentage of intracellular survival was calculated as described in the materials and methods. The survival of EHEC EDL933 wild-type (WT) was 0.22% immediately after the Gm-treatment and was reduced by 14-fold after 24 h of the Gm-treatment (Table I). Thus, EHEC can be internalized by amoebae and survive within *A. castellanii* cells, but they are not able to grow within them during the first 24 h. In contrast, only 0.02% of the *E. coli* K-12 inoculums were recovered immediately after the Gm-treatment and *E. coli* K-12 were not detected after 24 h of interaction with *A. castellanii*.

To investigate the roles of Stx and the T3SS (the two major virulence factors) in EHEC survival within *A. castellanii*, the strains EDL933Δ*stx* mutant (*stx1* and *stx2* isogenic double mutant) and EDL933Δ*escN* (non-functional T3SS) were included in the Gm protection assay. The results showed no significant changes in the survival of EDL933Δ*escN* mutant compared to the WT strain ($p > 0.05$). However, we found at least 3-fold more survival of EDL933Δ*stx* compared to the WT strain ($p < 0.001$) (Table I). Similarly to the WT strain, 8.4% of internalized bacteria of the EDL933Δ*stx* strain were recovered 24 h post-Gm treatment. Thus, the presence of Stx reduces the intracellular numbers of EHEC and their survival inside *A. castellanii*.

Post Gm-treated co-cultures using either EDL933 or EDL933Δ*stx* strains harboring GFP-plasmid were observed by confocal microscope. Not all amoebae contain viable bacteria and when EHEC were found within amoebae, we observed from 2 to 5 intracellular fluorescent bacteria per amoeba (Fig. 1). In addition, TEM observations of samples from the *in vitro* association assay showed one or many EHEC bacteria localized within amoeba phagosomes. We estimated about 10% and 30% of amoebae contained respectively EDL933 and EDL933Δ*stx* strains after 1.5 h co-culture (Fig. 2). In contrast, virtually no intracellular *E. coli* K-12 bacteria were observed.

Stx-toxins contribute to cell death and toxicity of *A. castellanii*

The fate of amoebae cells mixed and incubated with either EHEC or *E. coli* K-12 was compared. The direct counts of the total amoebae cells showed a significant increase of the percentage of dead amoebae at 1.5 h co-incubation with EDL933 (9.66%) compared to with *E. coli* K-12 (3.95%) ($p < 0.01$) (Fig. 3). In the absence of Stx, the results showed significantly

less mortality of amoebae after 24 h co-culture compared to the WT strain (EDL933 20.5%; EDL933Δ*stx* 15.5%) ($p < 0.05$). However, using TEM, we observed and estimated more than expected numbers of killed amoebae in the co-cultures with EHEC strains (data not shown). Dead amoebae were recognized by cell degeneration, loss of cytoplasm density and membrane integrity, and also nucleus dispersion.

The toxic effect of EHEC on amoebae was monitored by measuring levels of LDH released (Table II). Cytotoxicity of amoebae co-cultured 1.5 h with the EDL933 strains was significantly higher compared to with *E. coli* K-12 ($p < 0.01$). Further, after 24 h co-cultures, the toxic effect of strain EDL933 increased significantly compared to those seen with EDL933Δ*stx* mutant strain ($p < 0.01$) and *E. coli* K-12 ($p < 0.001$).

Discussion

In the present study, we examined the intracellular survival rate of EHEC O157:H7 using *A. castellanii* as the host model. We observed that *A. castellanii* can internalize EHEC. Internalized bacteria were able to survive within amoebae at least for a short period whereas *E. coli* K-12 (non-pathogenic control) cells were not. The *E. coli* K-12 strain HB101, in addition to the *rfb* rough mutation, also carries a *recA* mutation and thereby lacks the bacterial SOS response that could be involved in its reduced survival under stress conditions such as co-culture with a protozoan predator.

The results of the present study are compatible with a possible role of *A. castellanii* hosting and contributing to the environmental persistence of EHEC. This could be comparable to the role of *Acanthamoebae* species as hosts for *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Salmonella* Typhi in the aquatic environments of endemic areas [214,228,498,537,539]. Indeed, EHEC and *V. cholerae* share similarities being waterborne and causing diarrhoeal disease and generally are considered to be extracellular pathogens. Furthermore, like *V. cholerae* the EHEC in relation to *A. castellanii* apparently establish a controlled relationship with the amoebae and behaved as a facultative intracellular bacterium [540,541]. However, unlike EHEC, the presence of *V. cholerae* promoted the growth of amoebae and also enhanced growth, viability and survival of the bacteria [536,540]. In co-culture experiments, *S.* Typhi was not cytotoxic to *A. castellanii* and bacteria surviving in co-culture were extracellular and did not require contact with amoebae for their survival [537]. Hence, EHEC survival during

interaction with *A. castellanii* is quite distinct from survival strategies of other bacteria such as *S.* Typhi or *V. cholerae*. Interestingly, our results are similar to previous findings of EHEC entry and survival in human THP-1 macrophages [385,542]. In these studies, the EHEC *stx* mutant survived to a greater extent and was less cytotoxic to the macrophages than the WT parent strain. Moreover, it is of note that *Acanthamoeba* and human macrophages share morphological and functional similarities; especially in their phagocytic activity and parallel mechanisms in their interactions with many bacterial pathogens [216,386]. Consequently, *Acanthamoeba* has been suggested to be a key step in the evolution of environmental bacteria to become human pathogens. Thus *Acanthamoeba* may provide a useful model to study EHEC pathogenesis and to understand immune evasion mechanisms.

Using a similar approach but with a much higher multiplicity of infection (MOI 1000:1) and a shorter time of interaction (30 min), Carruthers *et al.,* have shown increased expression of the virulence genes including *stx* genes and a set of genes involved in T3SS components [380]. In our study, the role of Stx and T3SS for survival within *A. castellanii* was examined. We have shown that *stx* mutant but not *escN* mutant exhibited an increase in intracellular survival compared to the WT parent strain. This indicates a beneficial role of free-living amoebae for EHEC fate and survival in the absence of Stx. However, it is also possible that intracellular survival assay can be biased by the fact that amoebae ingesting large number of Stx+ cells are killed and could be washed away and only those ingesting less Stx+ bacteria are counted. Furthermore, the growth of amoebae was restricted in the presence of EHEC (Table III) and this could be at least partially linked to the toxic effect of Stx. Nevertheless amoebae in co-culture with Stx- strain are less killed resulting in higher counts of the Stx- strain per adherent amoeba. In fact, the Stx- strain was less cytotoxic to the amoebae than the wild-type strain. In short-time co-culture, the toxicity of EHEC on amoebae appears independent of Stx toxins. In addition, a basal cytotoxicity effect was also observed with non-pathogenic *E.coli*. This suggests that additional determinants other than Stx of EHEC are important in the interaction with *A. castellani*. However, during extended co-culture periods, the amoebae Stx toxins influence the level of cytotoxicity indicating a role of Stx in EHEC long term interaction with the amoebae. Lainhart *et al.* found that Stx-encoding bacteria killed the ciliate protozoa using the holotoxin Stx as an anti-predator weapon [517]. Other studies have shown that the presence of Stx-encoding prophage augmented the fitness of *E. coli* O157

in the presence of protozoa [388]. These authors found that the ratio of Stx+ to Stx− bacteria increased after 3 days of co-culture with *Tetrahymena thermophila,* a ciliate protozoan presents in the ruminant gut. In contrast, other investigators did not observe any advantage or disadvantage of Stx lysogenic phage within the rumen protozoa [518]. Thus, the contribution of Stx to bacterial survival when facing protozoa seems to be variable depending on the conditions of the challenge and the protozoan models used in the co-culture assay.

Currently, the exact role of Stx in EHEC interactions with amoebae remains unclear. The classical mechanism of Stx1 and Stx2 toxicity on mammalian cell involves binding of the B_5-subunits to the globotriaosylceramide receptors (Gb3), on the cell surface [543,544]. The holotoxin is internalized by receptor-mediated endocytosis and targets the endoplasmic reticulum via the Golgi by retrograde transport [545]. The A-subunit blocks EF 1-dependent aminoacyl tRNA binding, thereby inhibiting protein synthesis [517,546]. The B subunit has been suggested to mediate intake and/or trafficking of Stx in the killing of *T. thermophila* by Stx [547]. The exact mechanism by which these toxins influence the uptake and survival of *E. coli* O157:H7 and its cytotoxicity on *A. castellanii* remains unknown.

Altogether, our results indicate a potential role of the environmental phagocyte *A. castellanii* for hosting and contributing to environmental survival of EHEC. Here, we have investigated one possible step contributing to EHEC persistence by surviving within *Acanthamoeba.* Thus, considering the small number of EHEC required for human infection, understanding the molecular mechanisms used by EHEC to survive within amoebae may help in the identification of targets for new potential strategies to fight against their transmission to humans. In addition to being a potential host reservoir for EHEC, *Acanthamoeba* seems truly to be environmental ground training for EHEC when further facing macrophages and the immune system once transmitted to humans.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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Figures and legends

Table I. Survival of EDL933 wild type, Δ*stx* and Δ*escN* mutant strains

	Survival index (%)	
	1.5 h	**24 h**
***E. coli* strains**		
EDL933WT	0.22 ± 0.06	0.015 ± 0.002
K-12 HB101	$0.02 \pm 0.006^{***}$	$0.001 \pm 0.0005^{**}$
EDL933Δ*stx*	$0.65 \pm 0.13^{***}$	$0.055 \pm 0.022^{**}$
EDL933Δ*escN*	0.18 ± 0.10^{ns}	0.021 ± 0.006^{ns}

A gentamicin protection assay was used to assess *E. coli* survival within amoeba after 1.5 h and 24 h of co-incubation. There was significant survival of EDL933 compared to the *E. coli* K-12 HB101 control strain. The Stx mutant strain showed a 3-fold greater survival compared to the WT strain and there was no significant (ns) difference for the EscN mutant strain. (***$p < 0.001$, **$p < 0.01$)

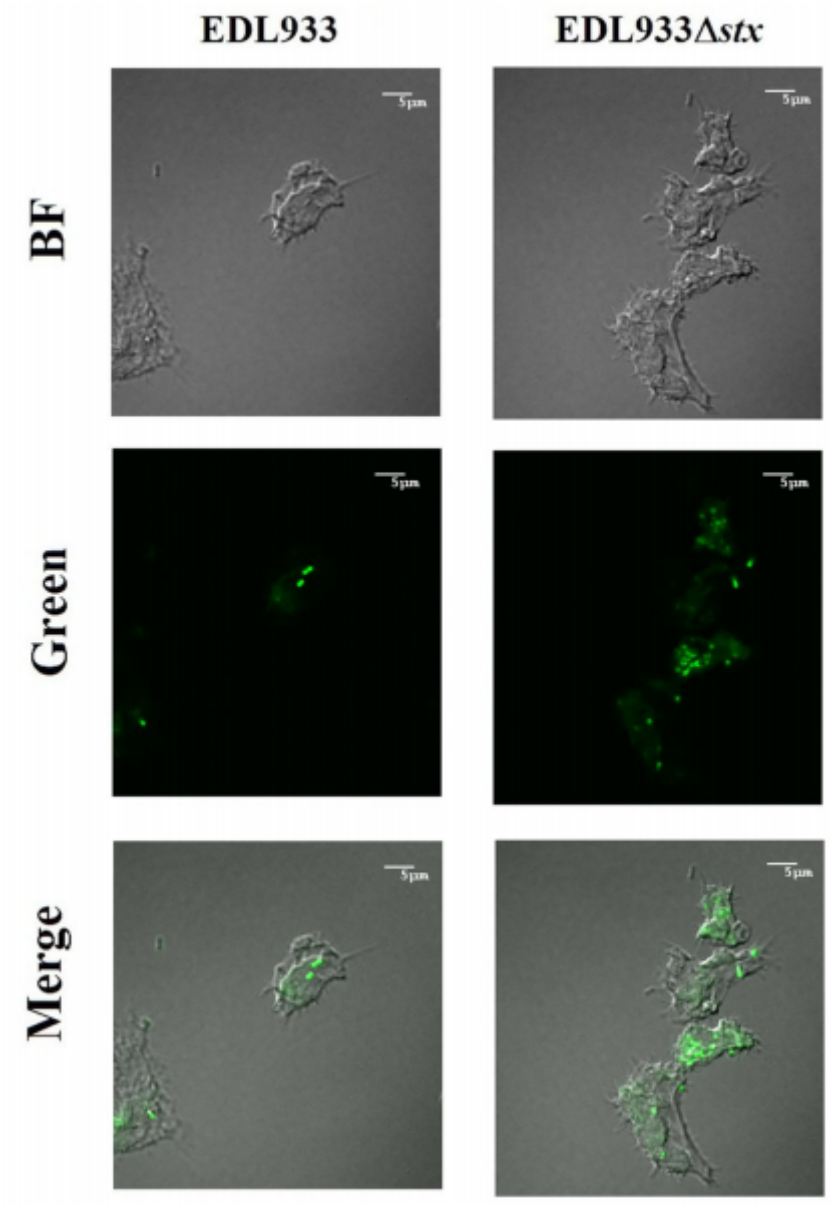

Figure 1. Fluorescence microscopic observations of GFP expressing EDL933 internalized by *A. castellanii*

Confocal fluorescence microscopy images were taken on Gm treated cells after 1.5 h incubation. The micrographs at high magnification (1000 x) show intracellular EDL933 strains expressing GFP (EDL933 strain in left panel, EDL933Δ*stx* mutant strain in right panel). Scale bars = 5 µm. BF= (bright field).

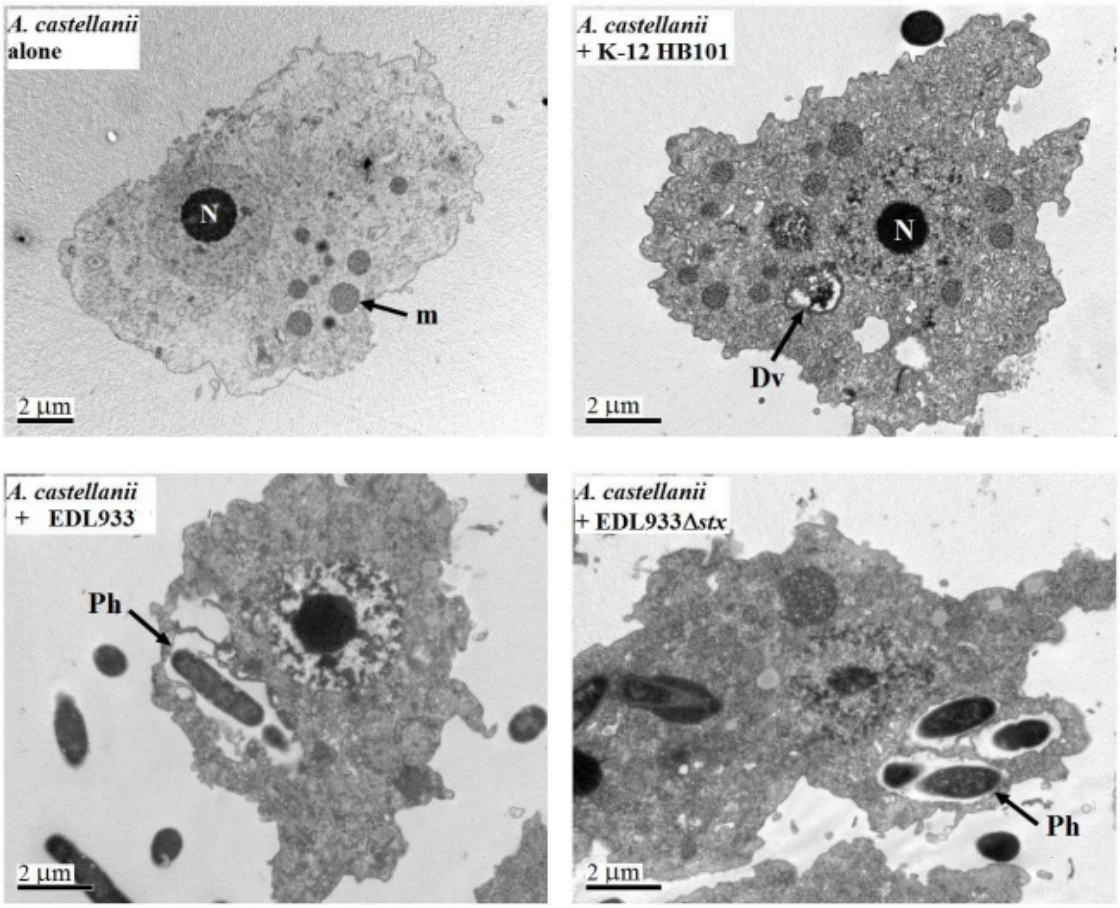

Figure 2. TEM observations of the intracellular localization of EHEC within amoebae

A. castellanii trophozoite in mono-culture (top left micrograph) and in co-culture with the *E. coli* K-12 control strain (top right micrograph), EDL933 strain (bottom left panel) and EDL933Δ*stx* mutant strain (bottom right panel). All micrographs represent 1.5 h incubation cultures. Ph = phagosomes containing one or many bacteria. Dv = digestive vacuole. N = nucleus. m = mitochondria.

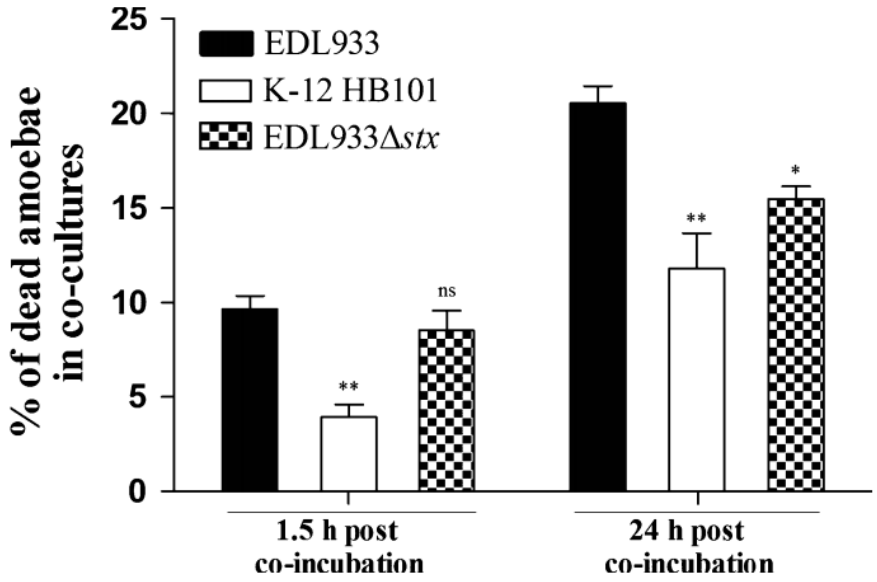

Figure 3. Percentages of dead amoebae cells during co-culture with *E. coli*

During the *in vitro* association assay, the direct count of the total amoebae cells showed a significant increase of amoebae mortality at 1.5 h post-infection with EHEC strains compared to with *E. coli* K-12. In the presence of EDL933Δ*stx,* amoebae death significantly decreases after 24 h co-incubation compared to that with the EDL933 parent strain. (*$p < 0.05$, **$p < 0.01$, ns: not significant $p > 0.05$).

Table II. The cytotoxic effect of EHEC on *A. castellanii*

	Percentage of cytotoxicity post co-incubation	
	1.5 h	**24 h**
***E. coli* strains**		
EDL933WT	25.3% ± 3.5	69.5% ± 5
K-12 HB101	16.0% ± 1.9*	32.1% ± 3.9**
EDL933Δ*stx*	20.3% ± 2.6 ns	40.9% ± 11.14**

The measurement of amoebae LDH release reflects the % of cytotoxicity that increases significantly in presence of EDL933 compared to K-12 at 1.5 h post co-incubation (*$p < 0.05$) and compared to either K-12 or EDL933Δ*stx* at 24 h post co-incubation (**$p < 0.01$), ns: not significant $p > 0.05$).

Suplemental data

Table III. The viable amoebae cell numbers in the presence of *E. coli*

	% of viable amoebae in co-cultures[a]	
	1.5 h	**24 h**
***E. coli* strains**		
EDL933WT	134.7 ± 10.4	167.7 ± 1.5
K-12 HB101	182.6 ± 7.5*	214.8 ± 3.7**
EDL933Δ*stx*	133.7 ± 8.4^{ns}	203.5 ± 8.6**

The initial inoculums of amoebae were 10^5 cells/mL. The viable number of amoebae cells increased significantly during 1.5 h co-cultures with *E. coli* K-12 compared to with EDL933WT. The co-incubation with EDL933Δ*stx* increases significantly the number of viable amoebae after 24 h compared to with WT parent strain. (*$p < 0.05$, **$p < 0.01$, ns: Not significant $p > 0.05$). [a]: Mean ± Sd of the following percentages = (Number of viable amoebae in co-cultures / The initial number of amoebae) x100.

Chapitre III : Étude du rôle du régulon Pho dans la virulence des EHEC

i. Article de recherche # 3. «PhoB active les facteurs de virulence chez les EHEC O157:H7 en réponse à la carence en phosphate»

Objectifs et approche utilisée

Cette étude avait pour objectif initial d'analyser la réponse transcriptomique globale de la souche EHEC EDL933 à une carence en Pi et à l'absence du régulateur PhoB. Suite à ces analyses, nous nous sommes focalisés sur la détermination de l'effet de carence du Pi et le rôle du régulon Pho dans l'expression des gènes de virulences notamment, les gènes du LEE codant le SST3 et certains de ses effecteurs ainsi que les gènes de Stx2 codés par le prophage BP-933W. Nous avons montré qu'en carence en Pi, la souche EDL933 sur exprime les gènes du LEE de manière PhoB-dépendante et PhoB-indépendante et augmente sa sécrétion de l'effecteur EspB. En parallèle, dans nos conditions expérimentales il y a augmentation d'expression, de production et de sécrétion de Stx2. De plus, PhoB serait capable de réguler directement les gènes du LEE et *stx2*. En effet, la protéine PhoB est capable de se lier *in vitro* à des sites spécifiques, boîtes Pho potentielles dont nous avons déterminé les séquences et les probabilités et qui sont situés dans les régions promotrices des opérons du LEE1, LEE2 et *stx2AB*.

L'étude a été réalisée en utilisant une approche multidisciplinaires regroupant des techniques de génétique bactérienne, de conditionnement bactérien au carence en Pi, d'hybridation sur puces d'ADN, de RT-PCR quantitative, de fusion avec gène rapporteur, de culture et infection de cellules HeLa, de test ELISA, des SDS-PAGE et western-blot, de production de protéines ainsi que le test de retard de migration électrophorétique (EMSA).

Contribution de l'étudiant

J'ai effectué presque la totalité des expérimentations. Grégort Jubelin a conçu le mutant EDL933 avec le système rapporteur de l'activité du promoteur *stx2*. J'ai aussi écrit et soumis cet article qui a été corrigé par mes directeurs et collaborateurs.

PhoB activates Escherichia coli O157:H7 virulence factors in response to inorganic phosphate limitation

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC), an emerging food- and water-borne hazard, is highly pathogenic to humans. In the environment, EHEC must survive phosphate (Pi) limitation. The response to such Pi starvation is an induction of the Pho regulon including the Pst system that senses Pi variation. The interplay between the virulence of EHEC, Pho-Pst system and environmental Pi remains unknown. To understand the effects of Pi deprivation on the molecular mechanisms involved in EHEC survival and virulence under Pho regulon control, we undertook transcriptome profiling of the EDL933 wild-type strain grown under high Pi and low Pi conditions and its isogenic Δ*phoB* mutant grown in low Pi conditions. The differentially expressed genes included 1067 Pi-dependent genes and 603 PhoB-dependent genes. Of these 131 genes were both Pi and PhoB-dependent. Differentially expressed genes that were selected included those involved in Pi homeostasis, cellular metabolism, acid stress, oxidative stress and RpoS-dependent stress responses. Differentially expressed virulence systems included the locus of enterocyte effacement (LEE) encoding the type-3 secretion system (T3SS) and its effectors, as well as BP-933W prophage encoded Shiga toxin 2 genes. Moreover, PhoB directly regulated LEE and *stx2* gene expression through binding to specific Pho boxes. However, in Pi-rich medium, constitutive activation of the Pho regulon decreased LEE gene expression and reduced adherence to HeLa cells. Together, these findings reveal that EHEC has evolved a sophisticated response to Pi limitation involving multiple biochemical strategies that contribute to its ability to respond to variations in environmental Pi and to coordinating the virulence response.

Keywords: EHEC EDL933, phosphate starvation, Pho regulon, PhoB, Pst system, transcriptomic, LEE, T3SS, Stx2.

Introduction

Two-component systems (TCSs) are signal transduction pathways commonly used by prokaryotes to sense and adapt to stimuli in the environment; as many as 50 different systems exist in bacteria, and at least 36 known TCSs are used by *E. coli* K-12 [402,548]. A typical TCS includes a histidine kinase (HK) and a partner response regulator (RR). In response to an input signal, the HK is auto-phosphorylated. Histidine-to-aspartate phosphotransfer to the RR results in transcriptional regulation and a cellular output response.

Inorganic phosphate (Pi) participates in many fundamental cellular processes [549]. In *E. coli*, the Pst system and the PhoB-R TCS coordinately mediate Pi sensing. The Pst system has two related but distinct functions: high-affinity uptake of Pi and the sensing of Pi levels regulating expression of the Pho regulon [406]. The *pst* operon (*pstSCAB–phoU)* encodes a transport complex belonging to the ABC transporter superfamily. The periplasmic protein PstS binds Pi, whereas PstA and PstC form a membrane channel. The ATPase PstB provides the energy for translocation and interacts with PstC [407]. PhoU has no evident role in Pi transport, but is required for control of the Pho regulon. In *E. coli*, there are at least 40 gene members of the Pho regulon that are primarily involved in phosphate assimilation and metabolism [341,403,404,550]. Transcription of all these genes is regulated in response to extracellular Pi concentrations via the TCS PhoB-R. During Pi limitation, the histidine-kinase PhoR phosphorylates PhoB. This in turn activates Pho regulon expression by binding to specific Pho box sequences located within the promoters of genes belonging to Pho regulon genes [404]. In addition, null mutations in *pst* genes disrupt the regulation of PhoB activation and lead to constitutive expression of the Pho regulon independently of environmental Pi availability [406]. Conversely, when Pi is replete, repression of the Pho regulon is mediated by an interaction between the Pst complex and PhoR, preventing PhoR-mediated phosphorylation of PhoB [551]. In these Pi-rich conditions, expressions of the Pst system becomes repressed and the Pit system induced. Reports indicate that in many bacteria, the Pi-limiting environment or the disruption of the Pst system, induce the Pho regulon and sometimes affect bacterial virulence [446,452,455,456,459,462,485].

Enterohaemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is an important pathogen that can cause a variety of clinical symptoms ranging from mild to severe bloody diarrhoea. The main virulence factors of EHEC are Shiga toxins (Stx), responsible for the hemorrhagic syndrome of the infection such as hemolytic uremic syndrome (HUS), and a T3SS through which EHEC translocates effector proteins into host cells, causing intestinal attaching and effacing (A/E) lesions [268,487]. The genes required for A/E lesions are encoded within a chromosomal pathogenicity island named the locus of enterocyte effacement (LEE) [552]. The LEE is composed of five major operons. The first gene of the LEE1 operon, *ler*, encodes a transcriptional regulator that positively regulates the expression of the LEE2, LEE3, LEE4 and LEE5 operons [553-556]. The LEE encodes the T3SS, an adhesin (the intimin Eae) and its receptor (Tir) required for intimate adherence to epithelial cells, and effector proteins translocated through the T3SS into the host cell. The complex regulation of LEE expression involves many positive and negative regulators [557]. Most of these factors influence LEE expression by directly or indirectly controlling *ler* expression; however, a variety of extra-transcriptional mechanisms have also been described that modulate the production of T3SS [558]. In EHEC, the genes encoding Stx1 and Stx2 are located in the genomes of lambdoid bacteriophages that can be induced from lysogenic strains. In the temperate state of the lambda (λ) phage, the CI repressor silences the transcription of most phage genes. The removal of repression that can occur when DNA damage activates the bacterial SOS response, leads to prophage induction [559,560].

EHEC is widely distributed in domestic ruminants, an important route for transmission to humans [489]. Cattle are the main reservoir for EHEC, especially serotype O157:H7 that is transmitted to humans primarily through consumption of contaminated foods. Faecal contamination of water and other foods may also lead to infection. EHEC can survive and persist in different ecological habitats such as soil, manure and aquatic environment (reviewed in [561]).

We have previously shown that during interaction between EHEC and the predacious protozoa *Acanthamoeba castellanii,* a free-living amoeba mostly found in aquatic environments, the expression of the Pho regulon contributed to adaptation to compete with amoebae in an environment that was limited in nutrients [460]. Indeed in

co-culture, the EDL933Δ*pst* strain reduced the growth of *A. castellanii* even more than the wild-type strain. This is also supported by the observation of an increase in expression of Pho genes in strain EDL933 when facing *A. castellanii* [562]. The Pho regulon is a global regulatory circuit involved in bacterial Pi management that has the ability to alter other cellular responses and virulence traits that affect bacterial survival when in association with amoebae in the environment [338,404]. In *V. cholerae,* the Pho regulon is required for *its* survival in both fresh water environments and the host small intestine [417].

EHEC, a facultative pathogen, thrives in nutritionally disparate conditions, bovine and human intestines, and in ecological habitats. The essential nutrient Pi is limited in some conditions such as the aquatic ecosystems and terrestrial environments. The concentration of Pi is usually low in natural environments [563,564]. The availability of Pi in the intestine is variable. It has been estimated from metabolic studies that in healthy adults consuming an average western diet, a Pi concentration of 15 to 30 mM is normally present under homeostatic conditions in the human intestinal lumen [565-567]. This level of *in vivo* Pi concentration should repress the Pho regulon. However, high level expression of some Pho regulon members (UgpB, PhoE, PhoA, Pst and PhoU) has been observed in EHEC O157:H7 isolated from piglet gut [568]. This indicates that EHEC cells can encounter Pi starvation signals in open environments as well as *in vivo.* Because we and others have observed that either Pi limitation or inactivation of the Pst system can modulate the virulence of intestinal and extra-intestinal bacterial pathogens [453-459,485,569], we hypothesized that EHEC virulence factors could be modulated in Pi-limited conditions.

Materials and Methods

Bacterial Strains and Culture Media

All strains used in this study are listed in Table 1. We used the EHEC O157:H7 strain EDL933 and its mutants Δ*phoB* and Δ*pstCAB* [460]. To measure the expression level of the *stx2* promoter, a genetic amplifier reporter system was used as described [570]. This

amplification system replaced the chromosomal coding sequence of *stx2AB* operon with the coding sequence of the T7 RNA polymerase gene (*T7pol*), resulting in strain EDL933Δ*stx2::T7pol*. This strain was next transformed with the multi-copy reporter plasmid pHL40 [570] that expresses *gfp* under the control of the T7pol-specific promoter. The Gfp background was determined in the EDL933Δ*stx* strain without *T7pol* fusion, also carrying the pHL40 plasmid. The fluorescence emission values (λ 520nm) were normalized with OD_{600nm} values. As a positive control for *stx2* transcription, 500 µg/mL of mitomycin C (MitC; the DNA-damaging agent) [571], was added to wild-type strain grown in high Pi during 3 h to induce the BP933W phage [572].

Pi Starvation, Culture Experiments and Alkaline Phosphatase Assay

EDL933 strains were grown at 37 °C in 3-(Nmorpholino) propanesulfonic acid (MOPS Teknova®) minimal medium [573] supplemented with 0.2% (w/v) glucose, thiamine (0.1 µg/mL), and indicated concentrations of Pi (K_2HPO_4). To test bacterial responses to Pi concentrations, cells from overnight LB cultures were inoculated into 25 mL of MOPS medium containing 1.32 mM KH_2PO_4 with a starting OD_{600nm} at 0.15 and grown to an OD_{600nm} of 0.3. Cells harvested from these fresh cultures were washed once and resuspended in 25 mL MOPS media containing either 1.32 mM (Pi-rich = Pi^+) or 1 µM (Pi-limited = Pi^-) KH_2PO_4. Growth was monitored during 16 hours by measuring OD_{600nm} and plating followed by CFU counts. For time-course monitoring of the Pho regulon expression, alkaline phosphatase (AP) activity was measured as described previously [456,485]. Briefly, aliquots were removed at indicated times, and 4 µg/ml of *p*-nitrophenyl phosphate (Sigma) was added to cells permeabilized by 50 µl of 0.1% SDS and 50 µl of chloroform. Color development was monitored at 420_{nm} and AP activity was expressed in Miller units (M.U.), and was calculated as follows: $1000 \times [OD_{420nm} - (1.75 \times OD_{550nm})] / T\,(\text{min}) \times V\,(\text{ml}) \times OD_{600nm}]$.

In MOPS Pi^- medium, the growth curve was similar between the wild-type and Δ*phoB* strains. However, the growth of the wild-type strain was delayed in Pi^- compared to in Pi^+. Therefore, we chose the comparison points based on similar CFU numbers (Figure S1A) and this was monitored by the measurement of PhoA activity (Figure. S1B).

RNA Extraction, Microarray Experiments and qRT-PCR

The EDL933WT strain and its isogenic Δ*phoB* mutant were grown in Pi^- and/or in Pi^+ conditions until the OD_{600nm} reached 0.55 to 0.6. Samples equal to 5 x 10^8 CFU were taken from this mid-log phase and processed for transcriptome analysis. Ten µg of RNAs were extracted and cDNAs were generated using reverse transcriptase. One µg of the fragmented and biotinylated cDNAs was hybridized onto Affymetrix GeneChip® *E. coli*. The data were processed using FlexArray® software using Robust Multichip Average (RMA) normalization procedure. The levels of transcription obtained from 3 biological replicates of each experimental condition were compared using the EB (Wright & Simon) algorithm. We then conducted comparisons between EDL933 grown in Pi^- and in Pi^+ conditions and between EDL933 and EDL933Δ*phoB* strains both grown in Pi^-. The differential expression conditions corresponded to a two-fold change (FC) cut-off and adjusted *p*-value < 0.05. Microarray results were validated by triplicate qRT-PCR. Each reaction was normalized to the *tus* housekeeping gene and the variation rate was calculated using the $2^{-\Delta\Delta Ct}$ method. All differential transcripts in both comparisons were functionally classified in COG according to TIGR's *Comprehensive Microbial Resource* [574].

Production of Constitutively Active PhoB ($PhoB^{CA}$)

The construction of the gene coding for the constitutively active PhoB ($PhoB^{CA}$) protein was based on previous work [575] in which the $PhoB^{CA}$ was obtained by the combination of two substitutions: Asp10Ala and Asp53Glu (DADE). $PhoB^{CA}$ was purified and produced by Genscript® as follows. Briefly, the $phoB^{CA}$ gene was cloned in a modified pGEX vector that contains a thrombin protease recognition site between the Glutathione S-Transferase (GST) tag and a multiple cloning site. Following the elution of $PhoB^{CA}$-GST using Glutathione Sepharose resin, the fused protein was cleaved by thrombin protease to remove the GST affinity Tag. The cleavage reaction was incubated again with Glutathione Sepharose 4B beads and the flow-through containing cleaved $PhoB^{CA}$ was collected. The protein concentration was determined by Bradford protein assay with BSA as a standard, and the purity was about 85% as estimated by densitometric analysis of the Coomassie Blue-stained SDS-PAGE gel.

Electrophoretic Mobility Shift Assays (EMSA)

The EMSA assay was adapted from previous reports [417,576]. The EMSA reaction mix consisted of $PhoB^{CA}$ at the desired concentrations (2.5, 5.0, 7.5 or 10.0 μM), 50 nM final probe concentration, 0.1 mg/mL calf thymus DNA and 0.1 mg/mL BSA in EMSA buffer (50 mM NaCl, 20 mM Tris, pH 7.4, 0.02% v/v sodium azide). Reactions were incubated for 30 minutes at 25°C, and then loaded onto an 8% native polyacrylamide gel running at 120V in 1x TBE buffer. Fluorescent bands were visualized using the Fusion Suite® imaging scanner. For each gene tested, the probes were amplified from the EDL933 genome using primers listed in Table S1. The forward primers used to generate all the probes contained 5′ 6-FAM fluorescein tags, to enable gel detection using a chemiluminescence scanner. The probes tested by EMSA assay represent candidate Pho-related virulence gene promoter regions that contain Pho box-sequences identified by *in silico* research of the EDL933 genome. To this end, we created a Pho Box matrix based on 12 Pho box-sequences from the known Pho-dependent genes in EDL933. The matrix frequencies were uploaded into the *Gibbs* algorithm tool at http://ccmbweb.ccv.brown.edu/gibbs/gibbs.html that browses the promoter query (Table S4).

Polyclonal EspB Antibody Production

We used a polyclonal anti-EspB serum obtained by rabbit immunization performed by Genscript (Piscataway, New Jersey, USA). Briefly, 2 rabbits were injected twice at 14-day intervals with a synthetic EspB antigenic peptide "CRRTQDDITTRLRDI" translated from the EDL933 genome. This peptide sequence was chosen according to its predicted antigenic potency and hydrophilic content, as determined by the Jameson and Wolf algorithm. After the second immunization, the serum titer was 1:512,000 as tested against the peptide by ELISA assay. The anti-EspB serum was then adsorbed overnight at 4 oC, at a 1:1 volume ratio against HB101 *E.coli* cell extract and incubated at 37 oC for 2 hours. Adsorbed material was eliminated by a 45-minute 45,000 rpm ultracentrifugation and 0.05% sodium azide was added to the antibody as a preservative. Unless otherwise indicated, all steps were done at 4 °C to prevent degradation.

Western-blot Analysis

Western-blot analyses were done as previously described [577,578]. Twenty ml of filtered culture supernatants that were normalized to CFU and OD_{600} and the total secreted proteins, were precipitated overnight with 10% of trichloroacetic acid (TCA) at 4 °C and then used to measure the secretion of EspB. Protein samples were resuspended in SDS sample buffer, boiled for 5 minutes, run on SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were probed with rabbit polyclonal antiserum against EspB (1:2000) and then with a goat anti-rabbit HRP-linked secondary antibody (Bio-Rad Laboratories, Hercules, CA). Proteins were detected with ECL-Plus horseradish peroxidase using the Western-blotting detection kit Promega®.

Determination of Stx2 Concentrations by ELISA

The production and release of Stx2B was quantified using the ELISA assay as described elsewhere [579]. The EDL933 strain was grown in MOPS/Pi+ or Pi-. Its Δ*phoB* derivative mutant and the complemented strain (harboring pHSG575-*phoBR*) were grown in MOPS/Pi-. Twenty mL cultures, normalized to OD600 0.6, were harvested and 8 mL of the supernatants were filtered and concentrated twice through a centrifugal filter (3KDa Amicon® Ultra-4) for 25 minutes at 3,700 xg. The remaining volumes were designed extra-cellular proteins and used to determine the concentration of secreted Stx2. The bacterial pellets were resuspended in 20 mL PBS, lysed by French press/sonication and then centrifuged for 20 minutes at 12,000 rpm. The lysate supernatants were designated whole cell proteins and used to access the amount of Stx2 production.

HeLa cell cultures and infections

The HeLa epithelial cell line was maintained in DMEM-HEPES with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C under 5% CO2. HeLa cells were seeded into 6-well plates (10^8 cells/well) and grown for 24 hours. Subsequently cells were washed, and 3 ml of fresh complete medium without antibiotics was added to the wells. Cells were infected with 10^9 bacteria per well for 5 hours. After 3 washes with PBS, cells were fixed using methanol for 15 minutes and stained with 10% Giemsa solution (Gibco®) for 20 minutes. The number of adherent bacteria per 25 cells was

determined by direct count under a light microscope (Magnification 640 x). The TTSS ΔescN strain [580] was used as negative control.

Microarray Accession Numbers

Microarray data have been deposited in the Gene Expression Omnibus database www.ncbi.hlm.nih.gov/projects/geo under accession number GSE50529 [NCBI tracking system #16869052].

Results

Effects of Pi Starvation and *phoB* Inactivation on the Transcriptomic Profiles of EDL933

EHEC strain EDL933 was grown in MOPS supplemented with low or high Pi at concentrations, respectively 1.0 μM and 1.32 mM. As shown in figure S1A, a decrease in growth rate was observed when the Pi concentration was limited to 1 μM. We also investigated the role of *PhoB* in EDL933 adaptation to Pi limitation using the Δ*phoB* mutant. The growth of the Δ*phoB* strain was similar to that of the wild-type strain in Pi-limited medium (Figure S1A). The alkaline phosphatase activity of PhoA is commonly used to evaluate the activation state of the Pho regulon [455]. As expected, the production level of PhoA in the wild-type strain was negatively correlated with Pi concentration. In addition, PhoA level was decreased over 100-fold in the Δ*phoB* mutant compared with the wild-type strain assayed in Pi-limited medium (Figure S1B). Thus, in the wild-type strain, the Pho regulon is highly activated during growth in Pi-limited medium and this activation is abrogated in the Δ*phoB* mutant.

Transcriptomic analysis was performed to identify the regulatory circuit of PhoB in EDL933. Complementary DNAs obtained from total RNA were hybridized onto Affymetrix GeneChip® *E. coli* 2.0 covering the entire EDL933 genome. Compared to the expression profile in high Pi medium, the expression of 1067 genes in the wild-type grown in low Pi medium were changed more than two-fold with an adjusted *P* value of <0.05 (Figure 1A) (Tables S1 and S2). We also compared the transcriptome of WT and Δ*phoB* strains cultivated in low Pi medium and found that 603 genes were differentially expressed.

Combining these two transcriptomic analyses, we classified genes into 3 distinct categories as shown in Figure 1A. Group 1 includes 936 genes with expression levels that changed in the wild-type strain between low and high Pi concentration, but did not change between wild-type and Δ*phoB* strains, therefore corresponding to PhoB-independent Pi response genes. In contrast, Group 3 includes 472 genes with expression levels that changed between wild-type and Δ*phoB* strains, but with no expression change in the wild-type strain grown in low or high Pi media, indicating that expression of these genes is PhoB-dependent, but not modulated by Pi concentration. Lastly, Group 2 includes 131 genes having expression modulated by both Pi concentration and by the PhoB regulatory protein (Table 2). The high proportion of genes that are differentially expressed between the Pi^+ and Pi^- regimes could be slightly affected by the difference in growth rate (Figure S1). To minimize that, equivalent CFU amounts were chosen from Pi^+ and Pi^- conditions that resulted in similar expression patterns of the Pho regulon members presented in Table 2.

The Cluster of Orthologous Gene (COG) classification was used to designate genes whose expression was altered during Pi starvation and was under the control of PhoB (Figure 1B). Upregulated genes include those encoding proteins of unknown function, transport and binding proteins, energy and central intermediary metabolism, and transcription (Table S2). Genes that were downregulated include the unknown function genes, and those involved with protein fate; protein synthesis; DNA metabolism; purine, pyrimidine, nucleoside and nucleotide pathways; and cell envelope proteins (Table S3). A large number of genes involved in stress responses and hypothetical proteins were also differentially expressed as were some virulence genes. In low Pi, there was a coupling between Pho regulon gene expression and the central pathways of carbon and energy metabolism, many of which are also formal pathways in Pi metabolism.

The expression patterns of the genome-wide transcriptional response that was observed shared similarities with transcriptomic analyses of *E. coli* K-12 during Pi starvation, and with the avian pathogen *E.coli* (APEC) and *Citrobacter rodentium* Δ*pst* mutants [403,456,459].

The global classification of differentially expressed genes in Pi limitation is shared with the effects of nutrient depletion. Some of these genes coordinate the

expression of hundreds of genes across a variety of cellular functions associated with cellular homeostasis and metabolism (Figure 1B). An expression of PhoB regulon genes was effectively induced in the wild-type strain grown in low Pi. In low Pi, the Pho regulon was downregulated in the Δ*phoB* mutant compared to the wild-type strain. Fold change for Pho regulon genes varied from 190.5- to 2.1-fold (Table 2). Besides those, in low Pi the *bssR* gene showed the highest induction (18.6-fold), whereas the greatest repression was observed for the *trpL* gene (9.9-fold) (Table II). Validation of microarray results was achieved using qRT-PCR. Comparison of gene expression by microarray hybridizations and qRT-PCR demonstrated a high level of concordance between the datasets, which is represented by a correlation coefficient of 0.87 in response to low Pi and of 0.82 in response to Δ*phoB* mutation (Figure S2).

Globally, the transcriptional profile of EDL933 grown in Pi-limited medium indicates that, in addition to up-regulating genes associated with scavenging pathways for Pi acquisition and conservation, many genes dealing directly with global stress were differentially expressed. Many lines of evidence suggest that the Pho regulon and the stress response are interrelated [338]. The Pho regulon and the RpoS regulon are interrelated regulatory networks of the bacterial adaptive response [338]. RpoS is a sigma factor implicated in the cellular response to many stresses. It is also implicated in the stationary phase and the induction of genes in nutrient-limiting environments [581]. The *rpoS* gene is expressed under a variety of growth conditions, but regulation and RpoS production is largely dependent on post-transcriptional stability [581,582]. In our study, the *rpoS* gene was not differentially expressed in strains grown in Pi-limited medium. However, in this condition the RpoS-regulatory gene *iraP* was induced (4-fold). IraP encodes an anti-adaptor protein that enhances RpoS stability and accumulation by inhibiting its targeting to the ClpXP degradosome [583,584]. Moreover, Mandel et al. has shown that RpoS translation and stability were significantly increased during Pi starvation [585]. This suggests that in our results the RpoS stability was probably increased in EDL933 grown in Pi-limited medium that could lead to regulatory expression of numerous RpoS-dependent genes. In addition, some genes involved in the oxidative stress and acid stress responses were found to be differentially expressed. Of these, some were modulated in the Δ*phoB* mutant such as *iraP, katE, ahpC, gadE* and

gadBC. These stress responses were also observed in the Δ*pst* mutant of APEC and *C. rodentium* strains [456,459].

Pi-limited Conditions Increased LEE Gene Expression in PhoB-dependent and Independent Manners

EHEC-specific virulence-associated genes were modulated in Pi-limited medium and in the Δ*phoB* mutant. Genes from LEE operons were 2.0- to 4.6-fold upregulated in the wild-type strain grown in Pi-limited medium compared to the wild-type strain grown in Pi-rich medium, and compared to the Δ*phoB* mutant (Fig. 2A). Moreover, several genes encoding LEE regulators such as *gadE* (+7.3), *pchA* (+7.6) and *ihf* (+2.6) were also upregulated in the wild-type strain in response to Pi starvation. However, in the wild-type strain compared to the Δ*phoB* mutant, genes encoding the non-LEE effectors NleB, NleE, NleH and NleG-1 and the LEE-negative regulator HN-S were upregulated (Tables S2).

We quantified the transcripts of *ler, sepZ, escV, espB* and *tir* encoded by LEE1, 2, 3, 4 and 5 respectively in the wild-type strain grown in Pi-limited and Pi-rich media and in Δ*phoB* strain grown in Pi-limited conditions. LEE-gene expression in the wild-type strain was 2.0-fold to 4.6-fold higher in response to Pi limitation (Fig. 2B). The tested genes that were downregulated in the absence of PhoB, *sepZ* and *escV* were the most significantly affected while the complementation of the Δ*phoB* mutant restored the *sepZ* and *escV* transcripts to wild-type levels (Fig. 2B). This suggests that PhoB is at least partially required for LEE activation in low Pi.

The EDL933 strain cultivated in Pi-limited medium delivers higher levels of extracellular proteins compared to when grown in Pi-rich medium (data not shown). Similarly, secretion of the effector protein EspB, encoded by LEE4, was more abundant in the wild-type strain grown in low Pi medium (EspB/GroEL ratio = 3.6 ±0.3) than in high Pi medium (1.2 ±0.3) and to a lesser extent in the Δ*phoB* mutant in Pi-limited medium (2.6 ±0.2) (Figure 2C). The Δ*phoB*-complemented strain showed a slight increase of EspB (2.8±0.4) compared to the mutant. These results showed that higher level of the EspB effector as detected in Pi-limited conditions, confirming that Pi limitation can increase LEE gene expression. Moreover Pi-dependent activation was partially PhoB-dependent.

In Pi-limited medium, PhoB Activated the Transcription, Production and Release of Stx2 and Controlled Gene Transcription of BP933W Regulators

Many BP-933W phage genes were upregulated in response to Pi starvation, including the anti-terminators N and Q, and the *stx2* and phage-late genes while expression of the CI repressor gene was downregulated (Figure 3A, Tables S2 and S3). In these Pi-limited conditions, genes encoding RecA and LexA, the SOS response marker and regulators, were not differentially expressed, while a gene encoding an indirect λ-phage regulator PcnB was repressed (Table S3).

In EDL933, Stx2 is encoded by *stx2AB* genes in the λ-like lysogenic phage BP933W. The *stx2AB* operon is located downstream of the late promoter region and upstream of the phage lysis cassette [162,586,587].

Measurement by qRT-PCR confirmed the significant decrease of *cI* expression and increase of *cro* and *stx2AB* genes in Pi-limited medium and in a PhoB-dependent manner (Figure 3B). As a positive control, MitC-induction of the BP933W phage resulted in high expression of *cro* and *stx2* genes while *cI* repressor gene expression remained at a basal transcription level. The Δ*phoB*-complemented strain exhibited similar levels of *cI* and *cro* transcription as the wild-type, although expression of the *stx2* genes was only partially restored. The transcriptional activation of *stx2* expression in Pi-limited medium was also observed using a GFP reporter system (Figure 3C).

To evaluate Stx2 synthesis, amounts of Stx2 were quantified in cells and supernatants using an ELISA assay. In response to Pi-limited conditions, the production and release of Stx2B were increased by 2.9-fold (Figure 3D). By contrast, Stx levels were decreased 2-fold in the Δ*phoB* mutant, whereas complementation of the Δ*phoB* mutant restored Stx2 level to that of the wild-type parent strain. These results demonstrate that the production of Stx2 toxin by the EDL933 strain is modulated by Pi levels and the PhoB regulatory protein.

PhoB Binds to LEE1, LEE2 and *stx2* Promoter Regions *in vitro*

The PhoB binding consensus sequence is defined as a 22-bp region of double-stranded DNA that consists of two direct repeats of 11 bp [588]. We performed ***in silico*** analysis to identify putative PhoB-binding sites associated with the LEE promoters (Figure 4).

Pho box consensus sequences with high probability were identified in the LEE1 and LEE2 promoter regions. In LEE1, a Pho box was located between the *ler* distal P1 and proximal P2 promoters [589] and two Pho boxes were identified in the LEE2 promoter region [590].

To investigate whether PhoB directly binds to the LEE promoters containing putative PhoB boxes, EMSA experiments were performed using $PhoB^{CA}$, a constitutively active PhoB mutant that activates Pho regulon promoters in the absence of phosphorylation. EMSA results showed PhoB dose-dependent shifts of the probes designed from the LEE1 and LEE2 promoter regions but not the LEE3 promoter region (Figure 5). As expected, the addition of unlabelled probes (1:1) reduced the amount of shifted 6-FAM labelled probes. This data indicates that $PhoB^{CA}$ binds specifically to the LEE1 and LEE2 promoter regions *in vitro*.

Four Pho box consensus sequences were found in the promoter of the *stx2AB* operon (Figures 4 and 5A). Using $PhoB^{CA}$, EMSA experiments demonstrated that PhoB was able to bind the *stx2AB* promoter region (Figure 5B). Indeed, the probe designed from the *stx2* promoter region shifted with a dose-dependent addition of $PhoB^{CA}$ and the addition of unlabelled control probe competed well with the 6-FAM-labelled probe. Although a potential Pho box was identified in the intergenic region between *cI* and *cro* that overlaps the P_R promoter, no shift was observed by EMSA (data not shown). Thus, the increased expression of *stx2* could be a direct effect of PhoB in Pi-limited medium. This would suggest that increased transcription of BP933W genes could result from direct and indirect effects of PhoB, and could also be due to the PhoB-independent effect of Pi-limited conditions.

Constitutive Activation of the Pho Regulon Decreased LEE Gene Expression and Reduced Adherence of strain EDL933 to HeLa Cells

To investigate the influence of the Pho regulon and PhoB on adherence, we evaluated the attachment of Δ*pst* and Δ*phoB* mutants to HeLa cells in DMEM cell culture medium, which is a Pi-rich medium. The Δ*pst* mutant was used because the inactivation of the Pst system results in constitutive expression of the Pho regulon. Thereby, the Δ*pst* mutant should mimic the behavior of the wild-type strain grown in Pi-limited conditions.

Accordingly, the EDL933 Δ*pst* mutant grown in Pi-rich medium demonstrated strong Pho regulon activation as indicated by PhoA activity (350 M.U.) (data not shown). However, expression of LEE genes in the Δ*pst* mutant was decreased, which was in contrast to the wild-type strain in response to Pi starvation, a condition that activates the Pho regulon (Figure 2B). Furthermore, secretion of the EspB effector protein was also reduced in the Δ*pst* mutant confirming the repression of LEE genes in this mutant (Figures 2B and 2C).

While the adhesion phenotype of the Δ*phoB* mutant to HeLa cells was similar to that of the wild-type strain (≈ 230 bacteria/25 HeLa cells) (Figures 6), the adhesion of the Δ*pst* mutant was reduced to a level similar to a Δ*escN* mutant, which lacks a functional T3SS (≈60 bacteria/25 HeLa cells) (Figure 6). Complementation of the Δ*pst* mutant restored the adhesion phenotype of the wild-type parent strain. With regards to LEE gene expression, our results indicate that a Δ*pst* mutant, in which the Pho regulon was highly and unduly activated (*phoB*/*tus* transcript ratio ≈ 127), did not reflect the behaviour of the wild-type strain responding to low Pi in which the Pho regulon was moderately activated (*phoB*/*tus* transcript ratio ≈ 10.5). Nonetheless, LEE gene expression and EHEC adherence properties are importantly affected in a Δ*pst* mutant, indicating that alteration of the Pho regulon modulates the transcription levels of genes belonging to this pathogenicity island.

Discussion

In response to Pi limitation, EHEC uses the Pho regulon, a specific regulatory network for phosphate acquisition. The two-component signal transduction system of PhoB-R plays a crucial role in inducing Pho regulon genes to adapt to Pi starvation. Herein, we show that during its adaptation to Pi limiting-conditions, EHEC EDL933 modulates gene expression associated with the acquisition and metabolism of Pi and with mechanisms of general stress responses. In addition, concomitant changes in expression of virulence genes occur. This is also observed in other pathogenic *E. coli* including *C. rodentium* that causes EHEC-like intestinal A/E lesions [459]. Changes in global gene expression underlying the overall physiological state of the cells in Pi limitation are also shared with other conditions of nutrient depletion. Some gene subsets responded particularly to Pi

limitation stress. The Pho regulon has been shown to contribute to the virulence of atypical enteropathogenic *E. coli* (EPEC) and *C. rodentium* through the response regulator PhoB [458,459].

During growth under Pi limitation, the absence of PhoB affected the expression of genes involved in Pi metabolism and genes involved in stress response and virulence. This indicates that PhoB plays an important role in the survival of EHEC under stressful Pi limiting conditions. Additionally, we show that PhoB is involved in EHEC virulence by regulating the expression of important virulence genes of the LEE and Stx2. Thus, we identified a novel role for PhoB as a transcriptional regulator of importance for both, adaptation to Pi availability and regulation of key virulence systems of EHEC.

Using transcriptional analysis and ELISA assays we showed that Pi-limiting conditions activate the expression and production of virulence genes encoding the LEE T3SS and some effectors and the Stx2 Shiga toxin. By regulating LEE and Stx2 expression, EHEC is able to control expression of major virulence genes under Pi limiting-conditions. The virulence modulation is partly due to PhoB, or PhoB-regulated gene(s), because a Δ*phoB* mutation affected the expression of some virulence genes in Pi-limiting conditions.

Microarray and qRT-PCR analysis demonstrated for the first time that low Pi-induced signals activated LEE gene expression. Both, low Pi and PhoB increased protein secretion by T3SS. PhoB was required to significantly increase the transcription of LEE2 and LEE3 while the transcription of LEE1 required a low Pi signal rather than PhoB. However, PhoB interacts directly with the promoters of LEE1 and LEE2 operons containing Pho boxes.

The regulation of LEE expression is complex and involves many positive and negative regulators [557]. Most of these factors influence LEE expression through direct or indirect control of *ler* expression. However, a variety of extra-transcriptional mechanisms have also been described that regulate LEE expression [558]. Our results suggest that PhoB could act on LEE gene expression through its binding to the *ler*/LEE1 promoter, which in return would activate the whole LEE. In addition, PhoB is involved in expression of LEE2 by direct interaction with its promoter region. However, it also possible that LEE regulators other than PhoB may also be involved in the fine tuning of

expression of LEE genes. Indeed, some positive regulators of LEE such as PchA, IHF and the LEE-negative regulator GadE were found to be upregulated in Pi-limited conditions. In addition, the silencer HN-S was repressed in the Δ*phoB* mutant.

In contrast, the EHEC Δ*pst* mutant showed a reduced expression of LEE and reduced adherence. This is also observed in *C. rodentium* that also harbors the LEE locus, where the expression of some T3SS genes and the ability to adhere and colonize were reduced in the Δ*pst* mutant [458]. In the Δ*pst* mutant, the Pho regulon is constitutively and more strongly activated than in Pi-limiting conditions. Indeed, strong activation of the *phoB* gene was found in the Δ*pst* background compared to in the wild-type strain in low Pi. This may reflect the super-shift in EMSA LEE1 and such high levels of phospho-PhoB may actually repress the expression of LEE genes. We have previously shown that the level of activation of the Pho regulon influenced the degree of attenuation of APEC [455]. Moreover, it has been shown recently that different Pi conditions have conflicting requirements of PhoB expression levels for optimal cell fitness [591,592]. Thus, our results suggest that the level of Pho regulon activation influences expression of LEE. In low Pi conditions, LEE is activated, while in the Δ*pst* mutant, LEE is repressed.

In the Sakai strain, Yoshida *et al.* [593], have demonstrated that the gene cluster esc0540-0544 is positively regulated by PhoB. This gene cluster is homologous to the *siiCA-DA* operon of *Escherichia fergusonii*, which encodes a putative RTX toxin and its cognate type I secretion system (T1SS) [593]. These findings taken together with our results suggest that PhoB acts as a virulence gene regulator in EHEC. Moreover, maintenance of proper regulation is critical, since both underexpression and overexpression can affect its virulence functions.

Signals from the host may arise within the intestine, such as changes in metabolite concentration, which allow bacteria to monitor infection and alter their behavior. Pieper *et al.* [568], have recently shown the activation of the Pho regulon of EHEC in the intestinal environment of gnotobiotic piglets. The signal that leads to induction of the Pho regulon is likely to be Pi limitation. However, we cannot be certain as PhoB can be regulated by a number of signals in addition to Pi concentration [419,549,594,595].

Many genes of the lysogenic λ phage BP933W were upregulated in response to Pi-limiting conditions including *stx2 w*hile the CI repressor gene was inhibited. The

repressor system of BP933W maintains the prophage in a quiescent state, [170]. Phage induction is a complex process dependent on bacterial and phage factors. Prophage induction, which leads to lytic growth, results from removal of repression. Ultimately the repressor controls Stx production and/or release. Stx2 production was increased in Pi-limited medium. Factors that regulate the switch between lysogeny and lytic growth, e.g., repressor, operator sites, and associated phage promoters, play important roles in regulating the production and/or release of Stx [587]. In Pi-limited conditions, PhoB indirectly inhibits the transcription of the repressor gene *cI*, which in return releases the constitutive transcription of *cro* that should induce a lytic phage cycle, thereby increasing *stx2* transcription. Prophage induction ultimately results in Q-modified transcription initiating at $P_{R'}$ that transcends the $t_{R'}$ terminator, leading to expression of downstream genes that include *stx2* and those encoding lysis functions. Another scenario could be direct PhoB binding onto sequences upstream of the *Stx2* promoter that activates *stx2* transcription.

Induction of the SOS response that commonly occurs through damage to the bacterial cell's DNA can lead to significantly higher levels of prophage induction. The SOS response refers to the production of a large number of enzymes following damage to DNA [176]. These enzymes include the activated form of RecA protein that facilitates auto-cleavage of many phage repressors and the bacterial LexA protein, which itself controls expression of the SOS response. In Pi-limited conditions, it appears that phage induction is RecA- and LexA-independent. RecA-independent induction also occurs in phages infecting *E. coli* [596-598]. In phage λ, induction does not always imply repressor cleavage by RecA. In some cases, the mechanism involves RcsA, a regulator of colanic acid synthesis, and DsrA [596,598], a small regulatory RNA that, among other functions, prevents the degradation of RcsA. Imamovic and Muniesa [599] showed that the Mg^{++} chelator EDTA affects the membrane structure of bacteria and triggers λ phage by RecA-independent mechanisms.

All together, these findings reveal that EHEC has evolved a sophisticated response to Pi availability involving multiple biochemical strategies that are likely critical to its ability to respond to changes in environmental Pi levels and also coordination of its virulence gene response by inducing LEE and *stx2* genes and controlling BP933W expression.

Tables and Figures

Table I. *E. coli* strains and plasmids used in this study

Strain/Plasmid	Description and relevant characteristics[a]	Source reference
EHEC strains		
EDL933WT	*E. coli* O157:H7; wild-type	[529]
EDL933Δ*pst*	EDL933; *pstCAB::Km*; Pho-regulon constitutive	[460]
EDL933Δ*phoB*	EDL933; *phoB::Km*; Pho-regulon negative	[460]
EDL933Δ*escN*	EDL933; *escN::Km*; non-functional T3SS mutant	[124]
EDL933Δ*stx*	EDL933; Δ*stx1; stx2::Km*	[579]
EDL933Δ*stx2::T7pol*	EDL933; *stx1::Km*; *stx2::T7pol-Cm*^{r}	This study
***E. coli* laboratory strains**		
χ7213	SM10λpir *ΔasdA4,* Km^r	[600]
Plasmid		
pAN92	pACYC184::pst operon Cm^r	[601]
pHL40	Reporter plasmid that expresses *gfp* under the control of a T7 RNA polymerase-specific promoter and Gm^r	[570]
pHSG575	Low-copy-number cloning vector	[602]
pHSG575-*phoBR*	pHSG575, *phoBR*$^+$ Cm^R Km^R	This study

[a] Km^r, kanamycin-resistant; Cm^r, chloramphenicol-resistant; Gm^r, gentamicin-resistant; Ap^r; ampicillin-resistant

Table II. Genes among upregulated and downregulated PhoB-dependent Pi response genes

Functional class and gene name	Known or predicted function	WT/P_i^- vs WT/P_i^+		WT/P_i^- vs Δ*phoB*/P_i^-	
		FC	*P* value	FC	*P* value
DNA metabolism					
mutY	Adenine DNA glycosylase	-5.45	2.33E-04	-2.19	1.01E-02
recG	ATP-dependent DNA helicase RecG	-3.02	8.04E-03	-3.46	5.12E-03
Purines, pyrimidines, nucleosides. and nucleotides					
gsk	Inosine-guanosine kinase	-2.31	4.99E-03	-2.15	9.62E-03
purT	P-ribosylglycinamide formyltransferase 2	-3.00	6.16E-03	-2.17	8.97E-03
Energy metabolism					
ppc	P-enolpyruvate carboxylase	-3.80	5.54E-04	-2.05	1.02E-02
Central intermediary metabolism					
phoA	Alkaline phosphatase (***phoA-psiF***)	131.70	2.48E-07	58.70	3.99E-07
gadB	Glutamate decarboxylase (***gadBC***)	10.46	9.58E-05	2.41	1.10E-02
amn	AMP nucleosidase	14.54	3.29E-06	5.24	9.48E-04
Cellular processes					
ahpC	Alkyl hydroperoxide reductase (***ahpCF***)	6.28	1.00E-03	11.17	1.07E-03
Amino acid biosynthesis					
trpL	Trp operon leader peptide	-9.94	1.16E-05	-2.48	4.22E-03
Cell envelope					
pgaB	Biofilm adhesin polysaccharide PGA export lipoprotein	6.74	2.47E-04	2.48	3.50E-02
slp	OMP induced after carbon starvation	2.85	4.70E-02	2.75	7.20E-03
fhuA	Ferrichrome outer membrane transporter	-4.37	6.48E-03	-2.51	1.80E-02
Protein fate					
hdeA	Stress response protein acid-resistance protein	4.49	7.13E-03	2.10	4.03E-02
iraP	Anti-RssB factor. RpoS stabilzer during Pi starvation; anti-adapter protein	3.96	3.63E-03	2.42	7.74E-03
Protein synthesis					
yibD	Predicted glycosyl transferase	80.87	1.56E-06	14.99	5.68E-05
Z4332	Cytotoxin Efa-1 involved in posttranscriptional regulation of T3S proteins	3.10	2.31E-02	2.29	9.88E-03
Transcription & regulatory functions					
phoB	DNA-binding response regulator (***phoBR***)	4.28	3.25E-03	13.81	4.71E-04
gadE	DNA-binding transcriptional activator	7.32	1.72E-03	2.09	4.31E-02
Transport & binding					
pstS	High-affinity P-specific transporter (***pstSCAB-phoU***)	47.48	8.46E-06	15.67	5.31E-06
phnC	Phosphonate transporter ATP-binding protein (***phnCDEFGHIJKLMNOP***)	81.45	1.45E-06	11.61	7.08E-05
ugpB	Glycerol-3-phosphate transporter subunit (***ugpBAECQ***)	7.92	5.46E-04	5.67	1.74E-03
phoE	Outer membrane phosphoporin protein E	40.39	1.09E-04	11.72	1.14E-03
malE	Maltose transporter periplasmic protein	-2.46	2.31E-02	-2.09	2.20E-02
fadL	Long-chain fatty acid outer membrane transporter	-2.66	3.56E-03	-2.38	5.88E-03
Virulence-related determinants					
espH	*Z5115* Effector translocated to the host cell membrane by the T3SS. LEE3 operon	2.42	4.89E-03	2.56	9.88E-03
escD	LEE-encoded T3SS component	2.17	1.19E-02	2.38	1.43E-02
espF	EspF / hypothetical protein. LEE4 operon	2.02	2.79E-02	2.28	3.15E-02
Unknown function. unclassified and hypothetical protein					
bssR	Repressor of biofilm formation by indole transport regulation	18.58	1.35E-03	4.86	6.47E-03
ygaW	Predicted inner membrane protein. stress-responsive	5.98	2.65E-04	3.43	6.47E-03
yfiD	Autonomous glycyl radical cofactor GrcA	5.58	1.29E-02	3.16	1.42E-02
psiE	P-starvation-inducible protein PsiE	20.81	1.59E-05	3.10	1.74E-03
cybC	Cytochrome b562	3.56	5.29E-04	2.01	1.41E-02
uspG	Universal stress protein UP12	6.89	1.81E-04	2.11	1.45E-02
yebE	Inner membrane protein. DUF533 family	5.19	5.75E-05	2.34	6.47E-03
yjeI	Conserved protein	3.72	3.01E-04	2.65	3.35E-03
matB	Cryptic Mat fimbrillin gene	2.60	9.23E-03	2.37	1.23E-02
ffs	Unknown non-coding RNA	11.99	4.59E-05	2.19	1.20E-02
Z2828	Predicted protein YoaI	5.68	1.61E-03	4.86	1.85E-03
Z3620	Hypothetical protein	7.24	4.79E-04	3.80	2.45E-03
yheT	Predicted hydrolase	-3.54	2.79E-03	-2.19	1.12E-02
yijP	Conserved inner membrane protein	-5.08	1.03E-03	-2.28	4.32E-02
yejL	Hypothetical conserved protein. UPF0352 family	-4.00	3.50E-04	-2.44	6.75E-03
yhcG	Hypothetical protein	-2.82	4.20E-03	-2.59	6.44E-03
ybdZ	Hypothetical protein	-3.74	2.13E-02	-2.76	6.75E-03
IG	Intergenic gerion *IG 696357_696735-r*	-2.21	1.61E-02	-2.88	8.87E-03

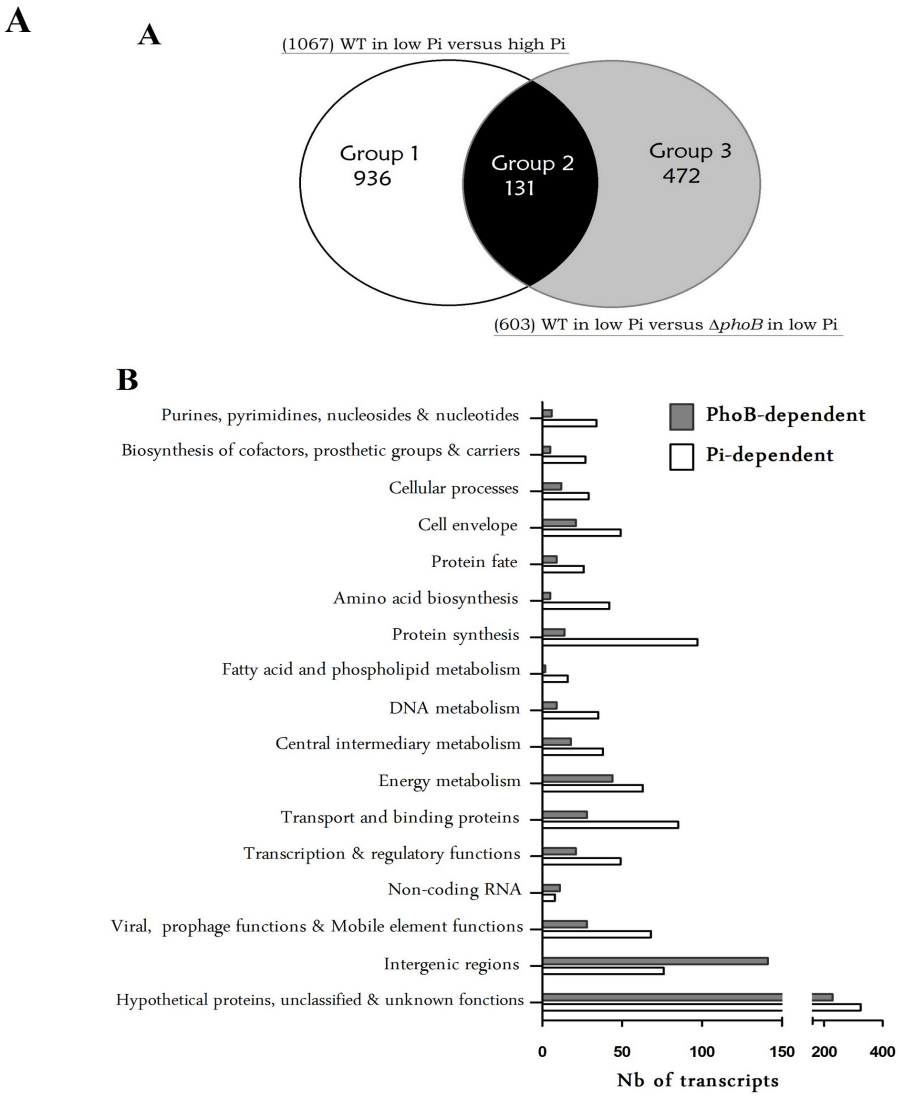


Figure 1. Global analysis of differentially expressed genes in response to Pi starvation or *phoB* inactivation in EDL933 strain

A. Classification of genes whose expression levels were altered in Pi-dependent and in PhoB-dependent manners. Left and right circles indicate the differentially expressed genes of wild-type and Δ*phoB*-mutant strains with expression levels that were altered over 2-fold under Pi limitation. Venn diagram: Group 1 includes 936 PhoB-independent genes that are differentially expressed under Pi limitation in the wild-type strain, but that did not change in the Δ*phoB* mutant. Group 3 includes 472 PhoB-dependent genes differentially expressed in the Δ*phoB* mutant but did not change under Pi-limitation in the wild-type strain. Group 2 included 131 PhoB-dependent Pi response genes that are differentially expressed under Pi limitation in the wild-type strain and between the wild-type and the Δ*phoB* strains. **B.** Functional classification of genes with altered expression in strain EDL933 grown in Pi-limited conditions compared to cells grown in Pi-rich conditions (Pi-dependent (white bars)) and EDL933 incubated in Pi-limited conditions compared to Δ*phoB* mutant cells grown in the Pi-limited conditions (PhoB-dependent (gray bars)).

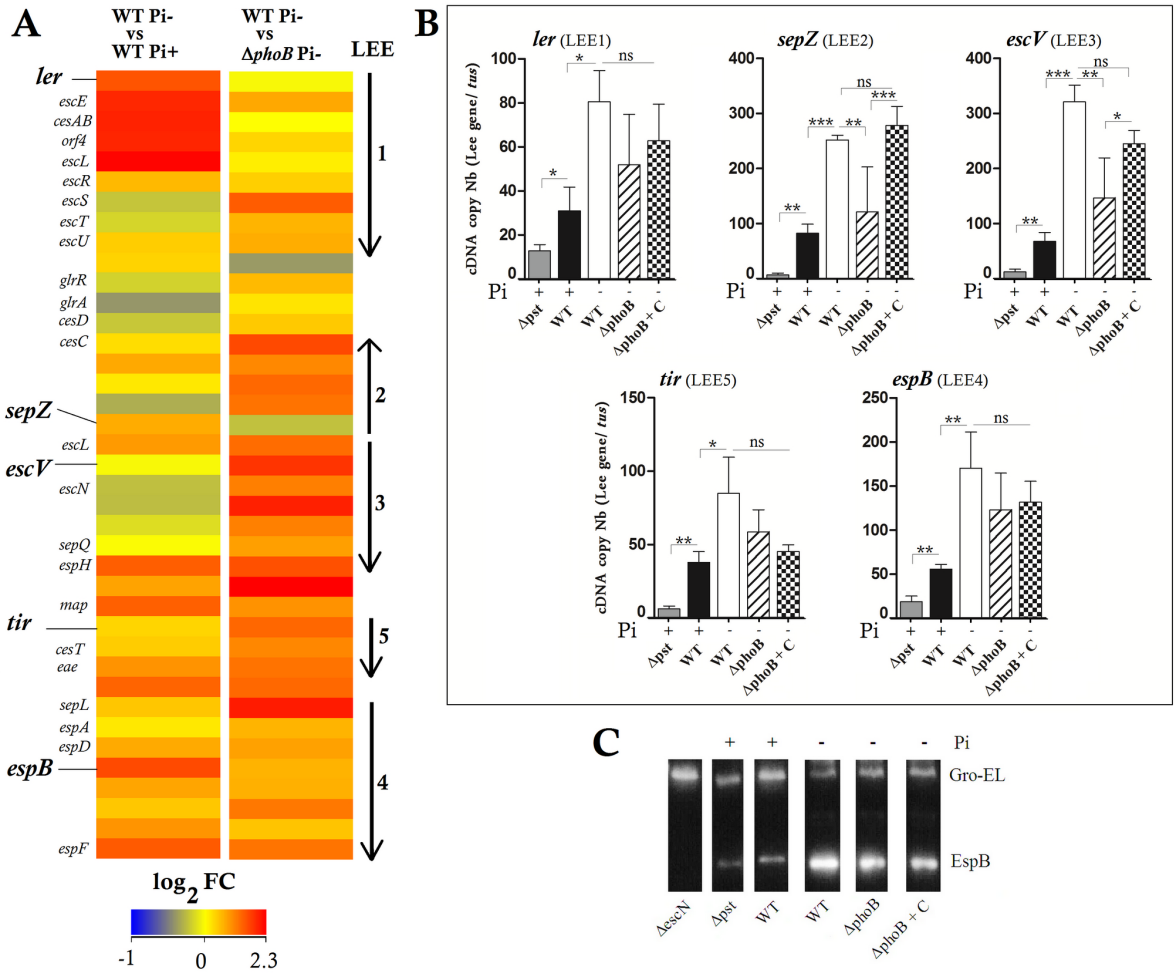


Figure 2. Low Pi conditions and deletion of *phoB* gene increased LEE gene expression and T3SS secretion of EspB in EDL933 strain.

A. A heat map of the expression levels of LEE genes between wild-type strain grown in low or high Pi concentrations, and between wild-type and Δ*phoB* strain grown in low Pi condition. Expression values were determined from the variance analysis by the EB (Wright & Simon) algorithm and are represented colorimetrically, with red representing up regulation (ratio of +2.3) and blue representing downregulation (ratio of -1) on a $\log_2$ scale. The data are represented as the means from three biological replicates. **B.** Expression level of LEE genes *ler, sepZ, escV, tir* and *espB* was analyzed by RT-qPCR in the wild-type, the Δ*phoB* mutant and its complement (Δ*phoB*+C) and the Δ*pst* mutant grown in low or high Pi media as indicated. **C.** Western-blot using anti-EspB antibody against the supernatant of the wild-type, the Δ*phoB* mutant and its complement and the Δ*pst* mutant grown in low or high Pi media as indicated. The Δ*escN* mutant grown in DMEM was used as a negative control. One µg/mL of Gro-El was added as a protein precipitation control. Asterisks represent the significant ANOVA *p* value (*<0.05, **<0.01, ***<0.001). **ns**: Not significant.

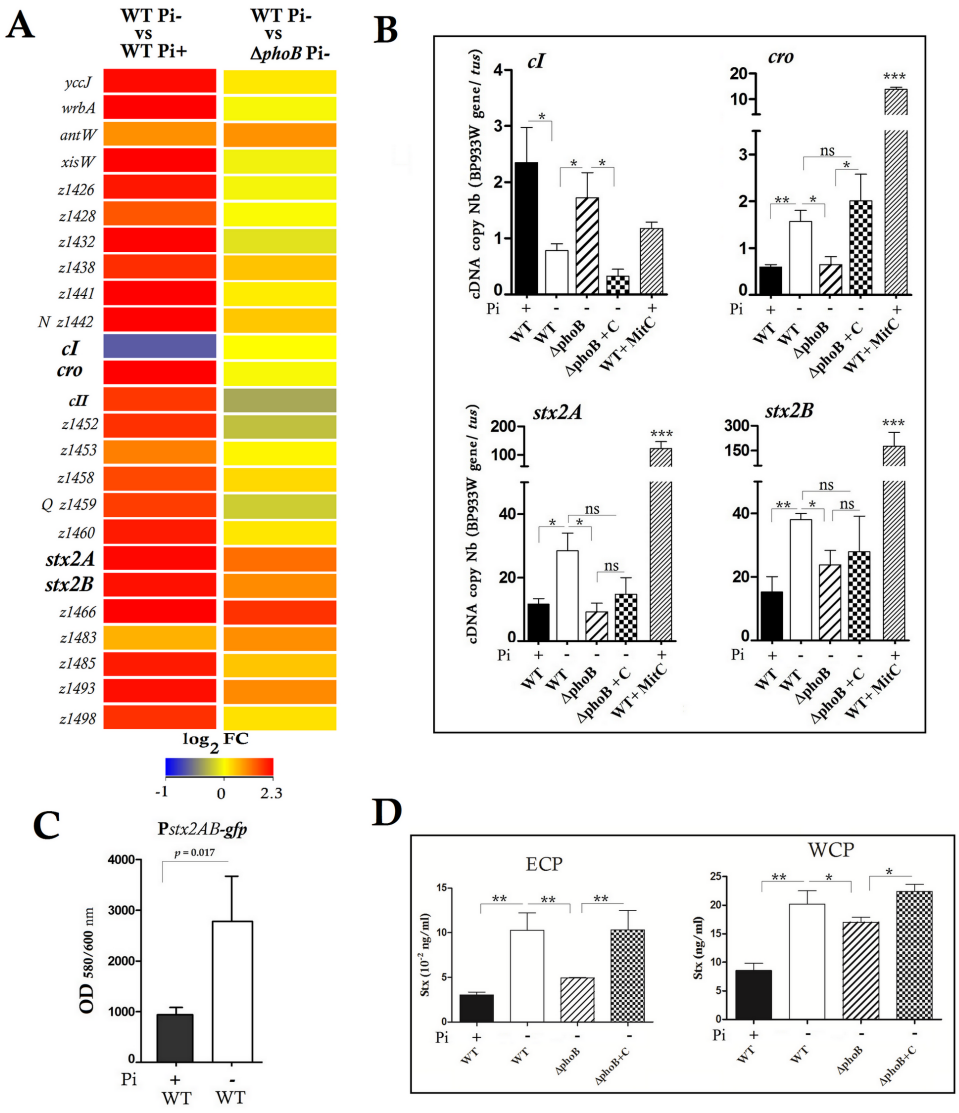
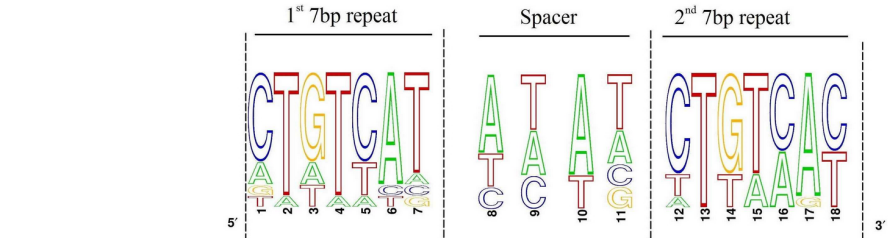


Figure 3. Effect of Pi and PhoB on *stx2* gene expression and toxin production.

Low Pi and PhoB increased transcription of *stx2* and its toxin production and of BP933W genes except for repressor gene *cI* that was repressed. **A**. Heat map of the expression levels of BP933W genes between wild-type EDL933 strain grown in low or high Pi conditions and between wild-type and Δ*phoB* strain grown in low Pi conditions. **B.** The expression levels of the BP933W genes *cI, cro* and *stx2AB* were analyzed by RT-qPCR in the wild-type strain and the Δ*phoB* mutant grown in low or high Pi media as indicated. The wild-type strain induced by mitomycin C (WT+MitC) was used as a positive control **C.** Fluorescence of the wild-type EDL933 strain carrying a chromosomal fusion reporting *stx2* transcription level grown in Pi+ or Pi- conditions. **D.** The production of Stx2 measured by ELISA in extra-cellular protein (ECP) and whole cell protein (WCP) fractions of the EDL933 wild-type strain grown in Pi+ or Pi- conditions and in the Δ*phoB* mutant and its complemented derivative. Asterisks represent the significant ANOVA *P value* (*<0.05, **<0.01, ***<0.001).



Promoter		Sequence				*p*
$P_{LEE\ 1}$	4689181..	C T A A T A G	CTTAAAAT	A T T A A A G	..4689159	0.98
$P_{LEE\ 2}$	4678461..	A T G T A C A	TTGCAACTA	T T T A A A T	..4678484	1.00
	4678403..	G T G T G A A	TATTATTG	C T T G A A A A	..4678426	0.96
P_{stx2}	1352036..	C C A T T A G	C T C A	T C G G G A C	AG ..1352056	0.98
	1352126..	T T G T T A G	C T C A	G C C G G A C	AG ..1352146	1.00
	1352154..	C C T T C T G	A G C A	A T C G G T C	AC ..1352174	0.77
	1352264..	A C T T C A G	C C A A	A A G G A A C	AC ..1352284	0.97

Figure 4. Bio-informatic analyses of PhoB-binding sites.

Sequence logos determined from 12 putative PhoB-binding sites in EDL933 are indicated (upper panel). Potential consensus sequences identified in the promoter regions of LEE 1, LEE 2 operons and upstream *stx2AB* genes are shown with their statistical scores and genomic positions (lower panel). Pho box prediction probabilities were determined using the matrix frequencies of Table S4 that were uploaded into the Gibbs software algorithm. http://ccmbweb.ccv.brown.edu/gibbs/gibbs.html

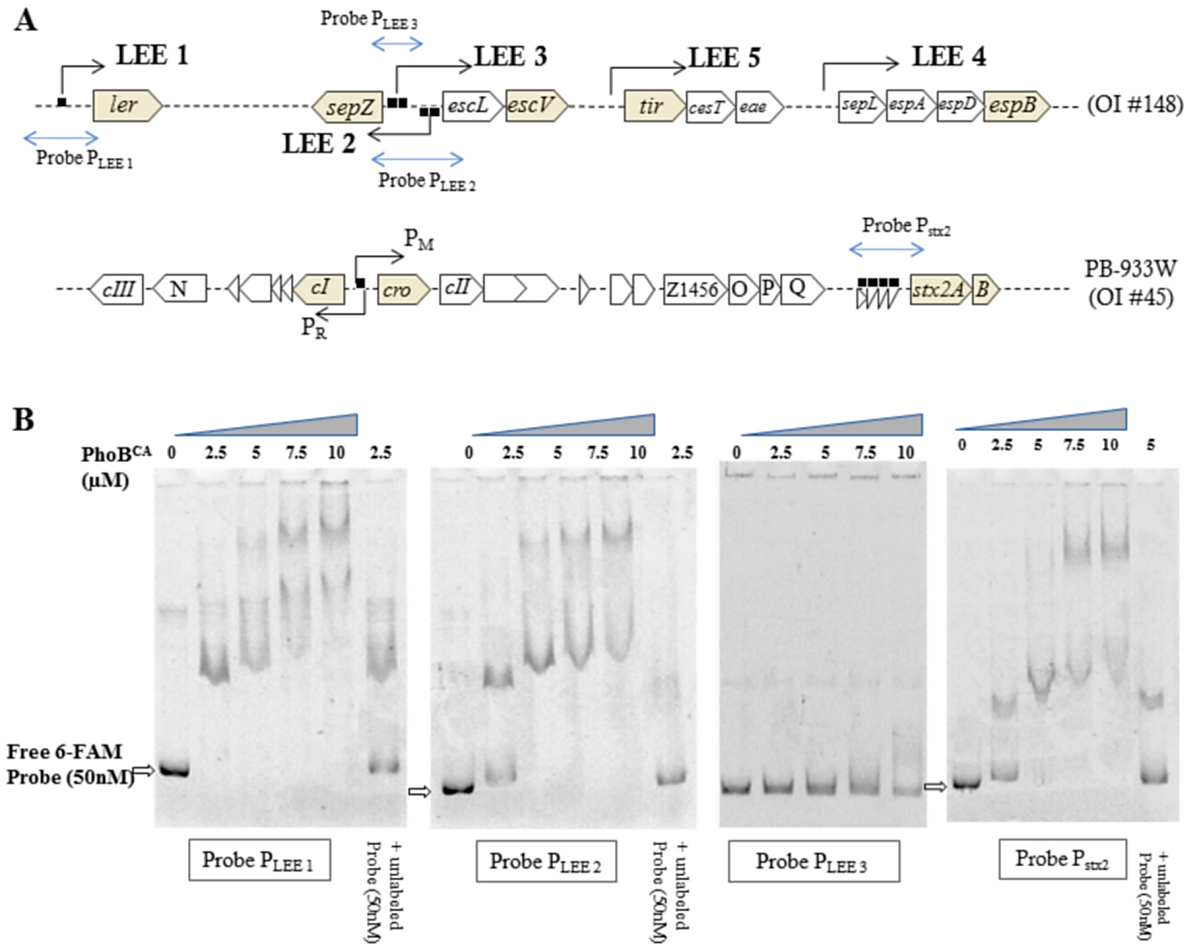


Figure 5. PhoB binds *in vitro* to LEE1, LEE2 and *stx2* promoter regions.

A. Schematic representation of LEE and lambdoid prophage BP933W DNA regions. Arrows indicate the orientation of transcription. The blue lines/arrows indicate the probes used for EMSA assays. The square symbol indicates the predicted Pho box. **B**. Increasing amounts of GST-purified recombinant PhoB^{CA} were used in the EMSA assay to shift the 6-FAM labeled DNA probes amplified from LEE1 (-253 to +64 bp), LEE2 (-228 to +81 bp), LEE3 (-109 to -259 bp) and *stx2AB* (-402 to -3 bp) promoter regions.

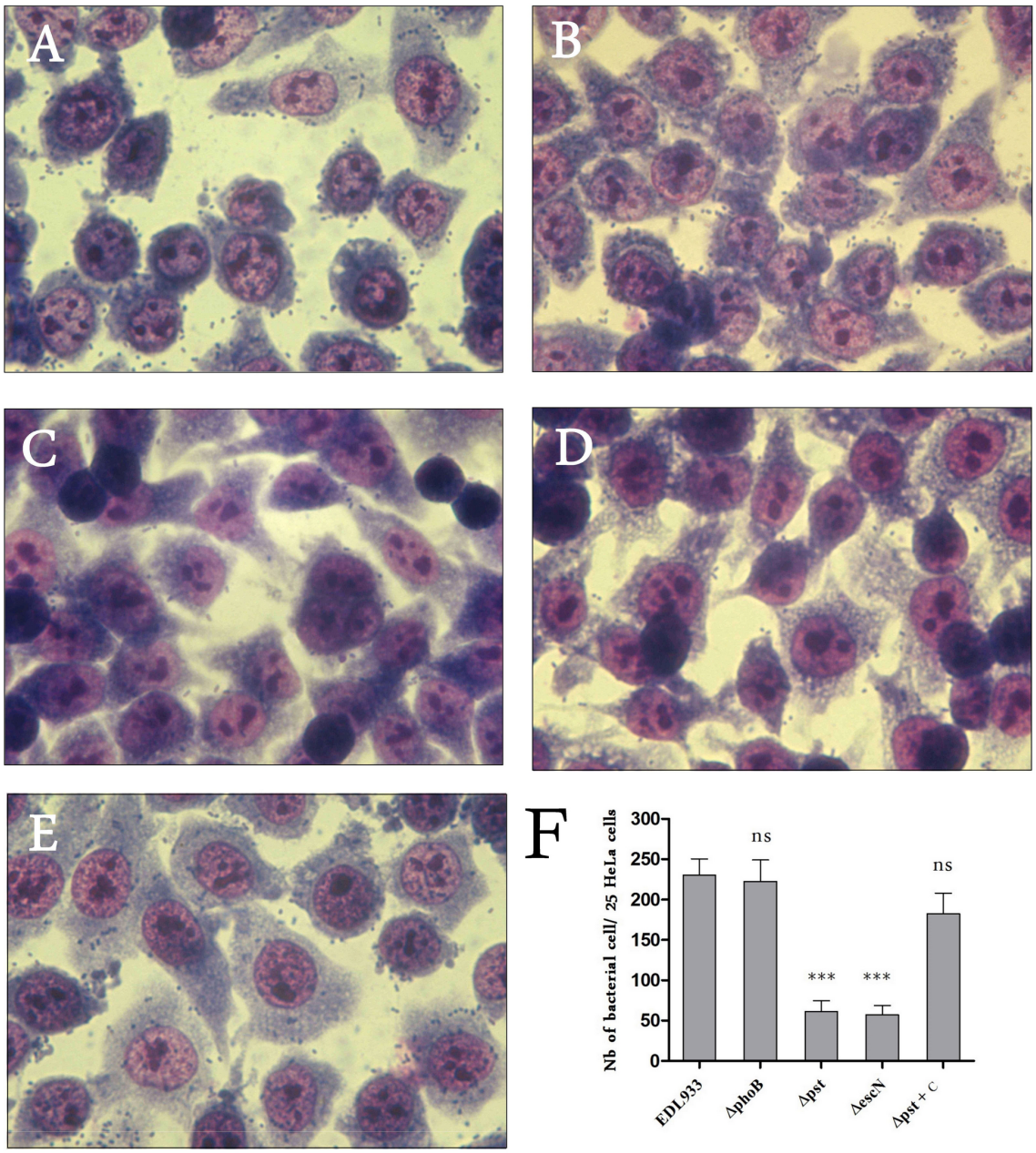


Figure 6. Regulation of adhesion of EDL933 to human epithelial cells by the Pho regulon.

HeLa cells were infected with EDL933 and indicated mutants. After 6 hours, cells were washed and colored with Giemsa. Adhesion to HeLa cells was similar between wild-type and Δ*phoB* strains (**A** and **B**). The adhesion decreased in the Δ*pst* mutant (P <0.001) to levels similar to that of the Δ*escN* mutant which lacks functional TTSS (**C** and **D**). The attachment phenotype was restored in the *trans* complemented Δ*pst* mutant (**E**). The number of adherent bacteria per HeLa cell was determined from 25 cells (**F**). Magnification 64 x.

Supplemental material

Table SI. Primers used in this study

Primers	Sequences 5' – 3'
***stx2* promoter fusion**	
stx2-T7polCam-F	CTGCGCCGGGTCTGGTGCTGATTACTTCAGCCAAAAGGAACACCTGTAT ATGCACACGATTAACATCGC
stx2-T7polCam-R	GTGACACAGATTACACTTGTTACCCACATACCACGAATCAGGTTATGCC ATGGAGTTCTGAGGTCATTACTG
Probes for EMSA	
P_{LEE1}- For-6Fam	GCTGAATGTATGGACTTGTTGT
P_{LEE1}-Rev	TGTTAACGAGATGATTTTCTTCT
P_{LEE2}- For-6Fam	AATCTTAAAAACTCTTCAACGT
P_{LEE2}-Rev	CTCATCCACTGAGTTATTTCCA
P_{LEE3}- For-6Fam	TTGCCTATGGGATAATTTGGTT
P_{LEE3}-Rev	CTCATCCACTGAGTTATTTCCA
P_{stx2}- For-6Fam	TAGTCAGTCAGAACGGATGAT
P_{stx2}-Rev	ACAGGTGTTCCTTTTGGCTGA

Table SII. Functional classification of upregulated genes

Functional classification of upregulated genes of microarray data comparing the wild-type strain grown low Pi to in high Pi (Pi-dependent) and comparing the wild-type strain to Δ*phoB* mutant both grown low Pi (PhoB-dependent).

Gene or Operon	Function and description	Microarray Fold Change [a]	
		Pi-dependent	PhoB-dependent
Transcription & regulatory functions			
phoB-R	DNA-binding response regulator in TCS with PhoR (or CreC)	4,28	13,81
phoU	negative regulator of PhoBR	12,27	8,56
gadE	DNA-bindingtranscriptional activator /acid-responsive regulator of gadA and gadBC	7,32	2,09
Z2382	Putative antitermination protein	2,25	--
adiY	Putative ARAC-type regulatory protein	2,81	--
ykgA	Putative AraC-like transcriptional regulator	2,15	--
Z0332	Ogr family transcription activator encoded in prophage CP-933I (OI #08)	2,34	--
Z2510	Putative transcriptional repressor (OI #70)	2,25	--
narL	transcriptional regulator	2,15	--
csgDE	DNA-binding transcriptional activator for *csgBA*	2,20	--
rpoH	RNA polymerase factor sigma-32	--	2,61
dhaR	DNA-binding transcription activator of the *dhaKLM* operon	--	2,19
yegE	Predicted diguanylate cyclase, GGDEF domain signaling protein	--	2,01
yjcT	D-allose kinase; NAGC-like transcriptional regulator	--	2,07
ygeV	Sigma-54-dependent transcriptional regulator	--	2,26
uhpA	DNA-binding response regulator in TCS wtih UhpB	--	2,46
hns	Global DNA-binding transcriptional dual regulator H-NS	--	2,56
ECs1091	putative transcriptional regulator (pchA)	7,55	--
Transport & binding			
pstSCAB	High-affinity phosphate ABC transporter	47,48	15,67
phoE	Outer membrane phosphoporin protein E	40,39	11,72
ugpBAECQ	Glycerol-3-phosphate transporter	7,92	5,67
napA	Nitrate reductase catalytic subunit	3,06	--
ftnA	Ferritin iron storage protein (cytoplasmic)	7,40	--
xylF	D-xylose transporter subunit	2,14	--
Z5691	Putative ATP-binding component of sugar ABC transporter	2,46	-2,60
Z5689	Putative periplasmic ribose-binding protein of ABC transport system	2,18	--

galP	D-galactose transporter	--	2,31
tnaB	Tryptophan permease TnaB	--	2,08
artJ	Arginine 3rd transport system	--	3,66
focA	Formate transporter	--	2,81
fecE	KpLE2 phage-like element; iron-dicitrate transporter	--	2,17
Energy metabolism			
ykgJ	Putative ferredoxin	2,06	--
frdD	Fumarate reductase (anaerobic), membrane anchor subunit	2,09	--
yliI	Soluble aldose sugar dehydrogenase	2,09	--
pflB	Pyruvate formate lyase I	3,29	--
gpmA	Phosphoglyceromutase 1	2,18	--
gadA	Glutamate decarboxylase alpha	6,96	--
gadBC	Glutamate decarboxylase beta, glutamic acid:γ-aminobutyrate antiporter	10,46	2.41
talB	Transaldolase B	2,18	--
pfkA	6-phosphofructokinase	2,35	--
yqeA	Carbamate kinase / predicted amino acid kinase	2,17	-2,24
ppsA	Phosphoenolpyruvate synthase	2,69	--
pgk	Phosphoglycerate kinase	--	2,21
dld	D-lactate dehydrogenase	--	2,60
glgA	Glycogen synthase	--	3,26
cydA	Cytochrome D ubiquinol oxidase	--	2,12
tdh	Threonine 3-dehydrogenase, NAD(P)-binding	--	2,04
ybhM	Conserved inner membrane protein	2.09	-2,22
Central intermediary metabolism			
phnCDEFGHIJKLMNO	Phosphonate transporter and metabolisme	81,45	11,61
phoA- psiF	Alkaline phosphatase/P-starvation-inducible prot	131,70	58,70
amn	AMP nucleosidase	14,54	5,24
gldA	Glycerol dehydrogenase	2,41	--
gst	Glutathionine S-transferase	2,50	--
Z4209	Aspartate/ornithine carbamoyltransferase family protein	2,34	-2,15
adhE	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	--	2.98
DNA metabolism			
mutH	Methyl-directed mismatch repair protein	2,07	--
ybfLD	H repeat-containing protein / predicted transposase	2,60	-2,30
ihfB	Integration host factor subunit beta	2,62	--
Z5187	Putative replicase	3,01	-2,13
Cell envelope			
pgaABCD	Required for PGA synthesis; an adhesin essential in biofilm	4,57	2,40
slp	OMP induced after carbon starvation	2,85	2,75
Z0024	Putative type-1 fimbrial protein	2,32	-2,10
lpxC	UDP-3-O-acyl N-acetylglucosamine deacetylase	--	2.27
yeaF	Scaffolding protein for murein synthesizing machinery	--	3,31
wzx	O antigen flippase	--	2,16
slyB	Putative OMP	--	2,06
fcI	fucose synthetase	-2,22	2,35
Z3198	GDP-D-mannose dehydratase	--	2,26
wcaJ	Predicted UDP-glucose lipid carrier transferase	2,04	-2,45
Cellular processes			
yggR	Predicted pilus retraction ATPase	2,50	-2,22
vgrE	Rhs element protein (OI #65)	4,47	--
ahpCF	Alkyl hydroperoxide reductase subunit C	6,28	11,17
uspF	Nucleotide binding protein in the class II universal stress protein family	3,48	--
uspA	Universal stress protein A , global response regulator	4,37	--
uspD	Universal stress protein UspD	3,55	--
slyA	Increases expression of HlyE by antagonizing the negative effects of H-NS	2,59	--
Z4326	Putative enterotoxin	--	2,69
ddg	lipid A biosynthesis palmitoleoyl acyltransferase	--	2,03
Protein fate			

yecA	Conserved protein, UPF0149 family	3,45	--
sohB	Predicted inner membrane peptidase; multicopy suppressor of htrA(degP	--	2,03
dnaK	Chaperone Hsp70, co-chaperone with DnaJ	--	2,11
gspD	General secretory pathway component, cryptic	--	2,13
pepP	Proline aminopeptidase P II	--	2,18
Protein synthesis			
ycgV	Partial putative adhesion protein	4,39	--
yibD	Predicted glycosyl transferase	80,87	14,99
Efa-1/Z4332	Putative cytotoxin, involved in posttranscriptional regulation of type III secreted prot	3,10	2,29
Z2257	Rhs element protein	2,07	--
arnA	Predicted barnase inhibitor	--	2.02
Amino acid biosynthesis			
ilvY	DNA-binding transcriptional dual regulator, positive regulator for ilvC	2,21	-2,43
hisD	Histidinol dehydrogenase	--	2.26
argCB	Acetylglutamate reductase- kinase	--	2,53
Fatty acid and phospholipids metabolism			
Z1139	Putative diacylglycerol kinase	2,21	--
fabF	3-oxoacyl-(acyl carrier protein) synthase II	--	2,37
Purines, pyrimidines, nucleosides, and nucleotides			
ugd	UDP-glucose 6-dehydrogenase	--	2,52
Z3513	Bifunctional UDP-glucuronic acid decarboxylase	--	2,02
Biosynthesis of cofactors, prosthetic groups, and carriers			
moaE	Molybdopterin guanine dinucleotide biosynthesis protein	2,30	--
dkgB	2,5-diketo-D-gluconate reductase B	8,92	--
gshB	Glutathione synthetase	--	2,05
yggC	Conserved protein with nucleoside triphosphate hydrolase domain	--	2,24
Viral, prophage functions, Mobile element functions (O islands, pathogenicity islands)			
xisW / Z1425	Putative excisionase for prophage BP-933W (OI #45)	4,80	--
Z1426	unknown protein encoded by bacteriophage BP-933W (OI #45)	3,63	--
Z1428	unknown protein encoded by bacteriophage BP-933W (OI #45)	3,05	--
Z1432	unknown protein encoded by bacteriophage BP-933W (OI #45)	3,16	--
gamW-exoW	host-nuclease inhibitor protein Gam of bacteriphage (OI #45)	3,35	--
Z1441	unknown protein encoded by bacteriophage BP-933W (OI #45)	4.98	--
Z1442	putative antitermination protein N of bacteriophage BP-933W (OI #45)	4,79	--
Z1448	regulatory protein Cro of bacteriophage BP-933W (OI #45)	4,76	--
Z1449	putative regulatory protein CII of bacteriophage BP-933W (OI #45)	2,62	--
Z1452	Unknown protein encoded by bacteriophage BP-933W (OI #45)	3,22	--
Z1453	Unknown protein encoded by bacteriophage BP-933W (OI #45)	2,05	--
ninG /Z1458	Unknown protein encoded by bacteriophage BP-933W (OI #45)	2,79	--
Z1459	Antitermination protein Q of bacteriophage BP-933W (OI #45)	2,21	--
Z1460	unknown protein encoded by bacteriophage BP-933W (OI #45)	3,08	--
Ant / Z1471	putative antirepressor protein Ant of bacteriophage BP-933W (OI #45)	3,33	--
Z1473	putative endopeptidase Rz of bacteriophage BP-933W (OI #45)	11,63	--
Z1483	putative tail fiber protein of bacteriophage BP-933W (OI #45)	2,48	--
Z1485	unknown protein encoded by bacteriophage BP-933W (OI #45)	3,26	--
Z1493	unknown protein encoded by bacteriophage BP-933W (OI #45)	3,35	--
Z1498	unknown protein encoded by bacteriophage BP-933W (OI #45)	4,00	--
Z1501	unknown protein encoded by bacteriophage BP-933W (OI #45)	3,16	--
xisN / Z1765	putative excisionase for prophage CP-933N (OI #50)	2,11	--
yafF / b1460	H repeat-containing protein	2,98	--
Z1782	unknown protein encoded by prophage CP-933N (OI #50)	4,96	--
Z1874	putative antiterminator Q of prophage CP-933X (OI #52)	2,02	--
Z1916	putative tail component of prophage CP-933X and cryptic prophage CP-933P	2,11	--
Z2079	unknown protein encoded in ISEc within CP-933O (OI #57)	2,39	-3,55
Z2122	putative holin protein of prophage CP-933O (OI #57)	2,44	--
Z6045	putative terminase encoded by prophages CP-933N (OI #50) & CP-933P	2,23	--

Z6065	unknown protein encoded by cryptic prophage CP-933P	2,21	--
ECs1648	putative tail fiber component J of prophages CP-933U (OI#79) & CP-933M (OI#44)	3,16	--
Z0984	unknown protein encoded by prophage CP-933K	2,04	--
Z0954	serine/threonin protein phosphatise encoded by prophage CP-933K (OI #36)	2,28	--
Z3073	unknown protein encoded within prophage CP-933U (OI #79)	2,49	--
ydcC / b1460	conserved protein	2,98	--
Z1135	complement resistance protein	2,89	-2,27
Z3354	putative exclusion protein ren of prophage CP-933V (OI #93)	2,92	--
Z3360	unknown protein encoded within prophage CP-933V	2,02	--
Z0953	NinG / unknown protein encoded by prophage CP-933K (OI #36)	2,03	--
Z0954	serine/threonin protein phosphatise encoded by prophage CP-933K (OI #36)	2,28	--
Z4313	putative pathogenicity island integrase	2,27	--
ECs1229	putative tail fiber protein of bacteriophage BP-933W (OI #45)	3,66	--
Noh / b0560	putative DNA packaging protein of prophage CP-933X (OI #52)	3,62	--
Z3929	unknown protein encoded by prophage CP-933Y (OI #108)	3,09	--
Z3938	hypothetical protein (OI #108)	2,67	-2,30
Z3128	putative inhibitor of cell division encoded within prophage CP-933U (OI #79)	2,68	-3,41
Z1326	putative inhibitor of cell division encoded by cryptic prophage CP-933M (OI #44)	2,09	-2,33
intQ	integrase fragment, cryptic prophage CP-933P/ intP_2	2,23	-2,51
Z1875	putative holin protein of prophage CP-933X	2,06	--
c_1462	putative tail component of prophage CP-933V	--	2,52
Z3269	Protein of Unknown function encoded in (OI #89)	3,31	--
hopD/ Z4693	putative leader peptidase (OI #130)	2,04	-2,17
Z4883	HicA-like protein (OI #139)	2,01	--
Ler / Z5140	LEE regulator encoded in lee1 operon (OI #148)	2,80	--
orf2 (escE)	LEE-encoded protein in lee1 operon (OI #148)	3,25	--
cesAB/ Z5138	LEE-encoded protein in lee1 operon (OI #148)	3,03	--
orf4// Z5137	LEE-encoded protein in lee1 operon (OI #148)	3,50	--
orf5/ Z5136	LEE-encoded protein in lee1 operon (OI #148)	4,58	--
sepD/Z5125	LEE-encoded protein in lee2 operon (OI #148)	--	2,25
escL/ Z5121	LEE-encoded protein in lee3 operon (OI #148)	--	3,32
escV/ Z5120	LEE-encoded T3SS membrane-associated ATPase, lee3 operon (OI #148)	--	2,15
escN / Z5119	LEE-encoded T3SS ATPase, lee3 operon (OI #148)	--	2,91
orf15/Z5118	LEE-encoded protein in lee3 operon (OI #148	--	3,35
orf16/Z5117	LEE-encoded protein in lee3 operon (OI #148)	2,42	2,56
espH/Z5115	LEE-encoded effector translocated to the host cell membrane by the T3SS, lee3 operon (OI#148)	2,42	3,41
cesF/Z5114	LEE-encoded chaperone (OI #148)	--	2,07
map/ Z5113	Type III secreted protein (OI #148)	--	2,18
tir /Z5112	translocated intimin receptor protein lee5 operon (OI #148)	--	2.50
eae/ Z5110	Gamma intimin adherence protein lee5 operon (OI #148)	--	2,36
escD/Z5109	LEE-encoded T3SS component (OI #148)	2,17	2,37
sepL/Z5108	LEE-encoded T3SS component, lee4 operon (OI #148)	--	4,11
espD/Z5106	LEE-encoded secreted protein EspD, lee4 operon (OI #148)	--	2,18
espB//Z5105	LEE-encoded secreted protein EspB, lee4 operon (OI #148)	2,13	--
escF/Z5103	EscF / hypothetical protein, lee4 operon (OI #148)	--	2,45
espF/Z5100	EspF / hypothetical protein, lee4 operon (OI #148)	2,02	2,28
nleB/ Z4328	Non-LEE-encoded effector protein (OI #122)	--	2,97
nleE/ Z4329	Non-LEE-encoded effector protein (OI #122)	--	2,53
nleH Z6021	Non-LEE-encoded effector protein (OI #71)	--	2,56
nleG-1/ Z2149	Non-LEE-encoded effector protein encoded in CP-933O, colonization factor (OI #57)	--	2,02
Non-coding RNA			
Ffs / b0455	4.5S RNA component of the signal recognition particle (SRP)	11,99	2,19
csrC / b4457	RNA inhibitor of CsrA	4,21	--
ssrS / b2911	6S RNA	3,59	--
rybA/ b4416	sRNA required for Mn homeostasis, under peroxide stress	2,90	--
gcvB / b4443	Regulatory sRNA requires Hfq and regulates expression of genes e.g. csgD	2,76	--
ryjA/ b4459	RyjA small RNA	2,43	2,40
rnpB/ b3123	catalytic subunit of RNAse P	--	4,90
gad/ b4452	GadY small regulatory RNA	--	2,90
rybB/ b4417	RybB small regulatory RNA	--	2,32

Table SIII. Functional classification of downregulated genes

Functional classification of downregulated genes of microarray data comparing the wild-type strain grown low Pi to in high Pi (Pi-dependent) and comparing the wild-type strain to Δ*phoB* mutant both grown low Pi (PhoB-dependent).

Gene/ operon	Pi-dependent	PhoB-dependent	Function and description [a]	Gene/ operon	Pi-dependent	PhoB-dependent	Function and description [a]
Transcription & regulatory functions							
rpoA	-6,33	--	DNA-directed RNA polymerase subunits	rhlE	-3,80	--	ATP-dependent RNA helicase
rpoBC	-7,06	--		dbpA	-3,48	--	
rpoD	-2,63	--		deaD	-2,88	--	
rpoZ	-3,46	--		pnp	-4,00	--	Polynucleotide phosphorylase/polyadenylase
rnc	-3,52	--	Ribonucleases	cusR	-2,41	--	Two-Component Signal Transduction System; DNA-binding response regulator and/or sensor kinase
rnpA	-14,81	--		phoPQ	-2,11	--	
rnb	-3,14	--		basRS	-2,35	--	
rnhB	-5,41	--		yehU	-4,48	--	
emrR	-3,23	--	DNA-binding transcriptional repressor	glnLG	-9,01	--	
purR	-5,55	--		cstA	-2,14	--	Carbon starvation protein
betI	-3,46	--		yjiY	-2,31	--	
dgsA	-3,00	--		cpdA	-2,89	--	3',5' cAMP phosphodiesterase
greA	-3,40	--	Transcription antitermination factors	cyaA	-2,19	--	Adenylate cyclase
nusA	-2,70	--		malT	-2,58	--	Transcriptional regulator
nusG	-2,07	--		pdhR	-2,16	--	
uhpC	-2,01	--	Membrane protein regulates uhpT expression	lrp	-2,16	--	
rbfA	-2,74	--	Ribosome-binding factor	ispH	-2,16	--	Diphosphate reductase
rnc	-3,51	--	RNase III/ role in degradation of RNA	mhpR	--	-2,10	Transcriptional activator, 3HPP-binding
fimZ	--	-3,32	Transcriptional regulator involved in fimbrial expression (LuxR/UhpA family)	yidL	--	-2,15	Predicted transcriptional regulator, AraC family
srmB	--	-2,85	ATP-dependent RNA helicase SrmB	ycjW	--	-2,03	Putative LACI-type transcriptional regulator
ybbS	--	-2,06	Transcriptional activator of the allD operon	ydeO	--	-2,25	Transcriptional activator for mdtEF
molR_ABD	--	-3,89	Putative regulators (fragment)				
Transport & binding							
livHMG	-4,02	--	Branched-chain amino acid transporter	aroP	-6,44	--	Aromatic amino acid transporter
cysPUWA	-3,23	--	Sulfate / thiosulfate ABC transporter	exbBD	-3,16	--	Biopolymer transport
ptsG	-3,57	--	PTS system glucose-specific transporter (glucose PTS permease)	glpFK	-4,51	--	Glycerol uptake channel and kinase

hisJQMP	-2,89	--	Histidine ABC transport	mglB	-3,80	--	Galactose-binding transport protein
potABC	-4,90	--	Putrescine / spermidine ABC transporter	manXY	-3,03	--	PTS system, mannose-specific (mannose PTS permeases)
metNIQ	-2,61	--	DL-methionine transporter	lysP	-5,45	--	Lysine transporter
msbA-lpxK	-2,73	--	Lipopolysaccharide ABC transporter	fepC	-3,37	--	Iron-enterobactin transporter ATP-binding protein
artPQM	-3,99	--	Arginine ABC transporter	putP	-2,75	--	Sodium/proline symporter
btuB	-3,58	--	Vitamin B12/cobalamin outer membrane transporter	gsiA	-2,09	--	Glutathione transporter
dctA	-3,45	--	C4-dicarboxylate transporter	dppB	-3,01	--	Dipeptide transporter
rbsDB	-4,14	--	D-ribose transporter	mtr	-2,78	--	Tryptophan transporter of high affinity
cusCFBA	-5,89	--	Copper / iron or silver efflux transport system	malEF	-2,46	-2,14	Maltose ABC transporter
copA	-3,48	--		lamB	-3,89	--	
trkA	-3,01	--	K+ transporter	oppB	-2,26	--	Oligopeptide transporter
trkH	-2,47	--		lptCAB	-2,25	--	Lipopolysaccharide transporter
yobA-yebZ	-2,48	--	Putative resistance proteins	lolCD	-2,42	--	Outer membrane-specific lipoprotein transporter
yjjK	-3,41	--	Putative ABC transporter ATP-binding protein	ydjN	-2,80	--	Predicted transporter /// part of a kinase
yheS	-2,29	--		fadL	-2,66	-2,38	Long-chain fatty acid outer membrane transporter
yddAB	-3,82	--		Z0463	-2,05	--	Hexosephosphate transport; putative response regulator
ptsA	--	-3,03	PEP-protein phosphotransferase system enzyme I	fepB	--	-2,62	iron-enterobactin transporter
fucP	--	-2,11	L-fucose transporter	agaV	--	-2,33	PTS system N-acetylgalactosamine-specific transporter
ygfU	--	-2,26	Putative purine permease	frvA	--	-2,23	Putative fructose-like phosphotransferase system
afuA	--	-2,02	Periplasmic ferric iron-binding protein	nanT	--	-2,40	sialic acid transporter
yabJ	--	-2,01	Thiamin transporter subunit	yjfF	--	-3,21	Inner membrane ABC transporter permease
nikA	--	-2,13	Periplasmic binding protein for nickel	Z5690	--	-2,20	Putative permease of ribose ABC transport system
Z2240	--	-2,47	IpaH-like protein	Z3023	--	-2,09	Putative secreted protein
Energy metabolism							
cyoABCDE	-7,48	--	Cytochrome o ubiquinol oxidase – heme O synthase	gcvTHP	-5,68	--	Glycine cleavage system
sucA	-2,20	--	2-oxoglutarate dehydrogenase, thiamin-requiring	speA	-2,82	--	Arginine decarboxylase
nuoABCEFGH IJKLM	-4,05	--	NADH dehydrogenase	atpIBEFH AGD	-4,30	--	ATP synthase F0F1 complex
gpsA	-4,07	--		fdx	-2,40	--	Reduced ferredoxin, electron carrer protein
pntAB	-3,25	--	NAD(P) transhydrogenase / pyridine nucleotide transhydrogenase	glpG	-2,24	--	Intramembrane serine protease
sdhCDAB	-10,68	--	Succinate dehydrogenase	lpdA	-2,58	--	Lipoamide dehydrogenase
aceEF	-4,60	--	Pyruvate dehydrogenase	glgX	-2,52	--	Glycogen debranching enzyme
ppc	-3,80	-2,05	Phosphoenolpyruvate carboxylase	maeA	-2,31	--	Malate dehydrogenase
acs	-3,23	--	Acetyl-CoA synthetase	gltA	-2,80	--	Type II citrate synthase
etp	-4,41	--	Phosphotyrosine-protein phosphatase	fdoG	-2,15	--	Formate dehydrogenase
wzxE	-2,58	--	Lipid III flippase, putative cytochrome	fldA	-2,25	--	Flavodoxin

poxB	--	-2,08	Pyruvate dehydrogenase	kdgK	--	-2,58	2-dehydro-3-deoxygluconokinase
adiA	--	-2,10	Biodegradative arginine decarboxylase	ydiST	--	-2,65	Predicted oxidoreductase ydiS
yihT	--	-2,18	Predicted aldolase	yfhL	--	-2,16	Ferredoxin-like protein ydiT, yfhL and
yhjN	--	-2,22	Regulator of cellulose synthase, cyclic di-GMP binding	yeiI	--	-2,35	Predicted kinase
sdaB	--	-2,06	L-serine dehydratase	ynfF	--	-2,43	S- and N-oxide reductase
ycbP	--	-2,37	NAD(P)H-dependent FMN reductase	araAD	--	-2,07	L-arabinose isomerise and L-ribulose-5-phosphate 4-epimerase
talc	--	-2,59	Fructose-6-phosphate aldolase	glpD	--	-2,16	Glycerol-3-phosphate dehydrogenase
hyfA	--	-2,11	Hydrogenase 4 Fe-S subunit	glpC	--	-4,04	
treC	--	-2,13	Trehalose-6-P hydrolase	hyaA	--	-2,06	Hydrogenase-1 small subunit
treA	--	-2,04	Periplasmic trehalase	hypE	--	-2,26	Hydrogenases maturation proteins
ygeX	--	-2,14	Diaminopropionate ammonia-lyase	fucK	--	-2,19	L-fuculokinase
lacA	--	-2,83	Galactoside O-acetyltransferase	srlR	--	-3,70	Repressor SrlR regulator for gut (srl), glucitol operon
putA	--	-2,11	Fused transcriptional regulator/proline andpyrroline-5-carboxylate dehydrogenase	astD	--	-2,01	Succinylglutamic semialdehyde dehydrogenase
malS	--	-2,78	Periplasmic alpha-amylase precursor	nrfG	--	-2,53	Formate-dependent nitrite reductase complex subunit NrfG
ascB	--	-2,16	Cryptic 6-phospho-beta-glucosidase	yihR	--	-2,65	Putative aldose-1-epimerase
ebgA	--	-2,04	Cryptic beta-D-galactosidase subunit alpha	yjeS	--	-2,40	Predicted Epoxyqueuosine reductase
tktB	--	-2,43	Transketolase	cadA	--	-2,06	Lysine decarboxylase, acid-inducible
Z5618	--	-3,10	Sorbitol-6-phosphate 2-dehydrogenase				
Central intermediary metabolism							
ureDABCE	-2,90	--	Ureases accessory and structural proteins	cysJI	-8,35	--	Sulfite reductase
rsxABCGE	-2,77	--	SoxR-reducing complex	gltBD	-5,67	--	Glutamate synthase
cysDNC	-5,44	--	Sulfate adenylyltransferases (cysDN) - adenylylsulfate kinase (cysC)	suhB	-2,30	--	Inositol monophosphatase
codA	-3,79	--	Cytosine deaminase	ygfT	--	-2,52	Putative oxidoreductase Fe-S binding subunit
DNA metabolism							
rep	-3,08	--	ATP-dependent DNA helicase Rep	gyrB	-3,10	--	DNA gyrase subunit B
holC- valS	-4,12	--	DNA polymerase III subunit chi - valyl-tRNA synthetase	rsmB	-3,42	--	16S rRNA methyltransferase B
priB	-26,77	--	Primosomal replication protein N	parC	-2,14	--	DNA topoisomerase IV subunit A
dnaAN-recF	-2,72	--	Chromosomal replication initiation protein - DNA polymerase III subunit beta - recombination protein F	xerD	-2,52	--	Site-specific tyrosine recombinase
				recO	-2,24	--	
nth	-3,06	--	Endonuclease III	dam	-2,64	--	DNA adenine methyltransferase
recQ	-2,01	--	ATP-dependent DNA helicase	dnaG	-2,99	--	DNA primase
recG	-3,02	-3,46		uvrC	-2,45	--	Excinuclease ABC subunit C
holD	-4,31	--	DNA polymerase III subunit psi	dnaE	-2,24	--	DNA polymerase III subunit alpha
dnaB	-6,28	--	Replicative DNA helicase	radA	-2,91	--	DNA repair protein

holA	-3,32	--	DNA polymerase III subunit delta	topA	-2,69	--	DNA topoisomerase I
mutY	-5,45	-2,19	Adenine DNA glycosylase	xseB	-2,69	--	Exonuclease VII small subunit
metK	-3,19	--	Methionine adenosyltransferase	gidB	-2,39	--	16S rRNA m7G527 methyltransferase
fis	-10,46	--	Fis DNA-binding transcriptional dual regulator	ruvC	-3,34	--	Component of RuvABC resolvasome, endonuclease
ybhP	--	-2,02	Conserved protein, endo/exonuclease/phosphatase family PFAM PF03372	holB	-4,19	--	DNA polymerase III subunit delta'
ybgA	--	-2,05	Conserved protein, DUF1722 family	yhdJ	--	-2,33	DNA adenine methyltransferase, SAM-dependent
polB	--	-2,92	DNA polymerase II	dinD	--	-2,30	DNA-damage-inducible protein D
Cell envelope							
murA	-3,54	--	UDP-N-acetylglucosamine enolpyruvoyl transferase	mepA	-2,86	--	Murein DD-endopeptidase
mrcA	-2,53	--	Peptidoglycan biosynthesis	mltC	-4,04	--	Murein transglycosylase
mreBCD	-2,47	--		asd	-2,31	--	Aspartate-semialdehyde dehydrogenase
murFXDGC	-3,76	--		nlpA	-2,25	--	Cytoplasmic membrane lipoprotein-28
waaFCL	-2,38	--	LPS biosynthesis enzyme	fcI	-2,22	--	Fucose synthetase
waaQGP	-2,91	--	LPS core biosynthesis protein	dacB	-2,44	--	D-alanyl-D-alanine endopeptidase
wzzE	-3,14	--	LPS biosynthesis protein (ECA)	fhuA	-4,37	-2,51	Ferrichrome / phage / antibiotic outer membrane porin
rlpB	-4,07	--	LPS-assembly lipoprotein	ddlB	-2,51	--	D-alanine--D-alanine ligase
mrdAB	-2,42	--	Cell shape; peptidoglycan synthetase; penicillin-binding protein 2	Z2200	-3,28	--	Putative major fimbrial subunit
lnt	-4,26	--	Apolipoprotein N-acyltransferase	Z2201	-3,02	--	Putative fimbrial chaperone protein
rffEDGHC	-3,40	--	UDP-N-acetylglucosamine epimerase/mannosaminuronic acid dehydrogenase dTDP- glucose dehydratase/pyrophosphorylase/fucosamine acetyltransferase	bamC bamA	-3,56 -3,26	-- --	OMP Assembly Complex
lpxDAB	-2,73	--	Acetylglucosamine acyltransferase	yqhH	--	-2,27	Puter membrane lipoprotein, Lpp paralog
htrE	--	-2,52	Putative outer membrane usher protein	flgE	--	-2,31	Flagellar hook protein
hofC	--	-2,14	Type IV pilin biogenesis protein	ybiP	--	-2,04	Predicted hydrolase, inner membrane
ppdD	--	-2,89	Putative major pilin subunit	Z0257	--	-2,22	Lipoprotein
Z5223	--	-2,22	Putative fimbrial chaperone				
Cellular processes							
cspG	-5,26	--	Cold shock protein	tolQRA	-2,79	--	Tol-Pal Cell Envelope Complex membrane spanning protein in TolA-TolQ-TolR complex
cspH	-8,41	--					
xerC	-3,03	--	Site-specific tyrosine recombinase	mukFE	-2,01	--	Chromosome condensins subunits
surE	-5,32	--	Stationary phase survival protein	ybeX	-4,66	--	Predicted ion transport protein
cvpA	-15,76	--	Colicin V production protein	ubiB	-2,62	--	2-octaprenylphenol hydroxylase
ybhC	-3,02	--	Acyl-CoA thioesterase, lipoprotein	rlmE	-2,32	--	23S rRNA methyltransferase
ftsW	-3,18	--	Essential cell division proteins	cafA	-2,42	--	Ribonuclease G
ftsY	-2,52	--		aldA	-2,62	--	Aldehyde dehydrogenase
ftsLI	-2,04	--		soda	-2,06	--	Superoxide dismutase

ftsN	-2,65	--		ybeZ	-2,02	--	Putative ATP-binding protein in pho regulon
flgN	--	-2,26	Flagellar export chaperone for FlgK and FlgL	emrY	--	-2,00	Multidrug resistance protein Y
fliK	--	-2,14	flagellar hook-length control protein	fimG	--	-2,06	Minor component of type 1 fimbriae
flgCJ	--	-2,24	Flagellar basal body rod protein FlgC and rod assembly protein/muramidase	ampC	--	-5,12	Beta-lactamase
fliLM	--	-2,22	Flagellar basal body-associated protein FliL and motor switch protein FliM	Z2263	--	-2,69	Rhs element protein
Protein fate							
ycbZ	-3,84	--	Putative ATP-dependent protease	pcm	-2,36	--	Protein-L-isoaspartate O-methyltransferase
secY	-9,84	--	Preprotein translocase subunit	bamB	-2,40	--	Lipoprotein required for OM biogenesis
secB	-3,22	--		surA	-3,15	--	Peptidyl-prolyl cis-trans isomerase
secDF	-4,85	--		prfC	-3,40	--	Peptide chain release factor 3
secG	-2,78	--		yibP	-2,16	--	EnvC murein hydrolase
hflKC	-3,19	--	Modulators for HflB protease specific for phage lambda cII repressor	pqqL	-3,37	--	Zinc protease
prc	-3,32	--	Mutational suppressor of prc thermosensitivity, outer membrane lipoprotein	rsmF	-4,62	--	16S rRNA m5C1407 methyltransferase
pepA	-5,27	--	Leucyl aminopeptidase	lon	-3,00	--	DNA-binding, ATP-dependent protease La
pepQ	-2,02	--	Proline dipeptidase	tig	-3,58	--	Trigger factor; a molecular chaperone involved in cell division
ylbB	-2,39	--	Allantoate amidohydrolase	groS	-2,84	--	Co-chaperonin GroES
dsbC	-2,37	--	Protein disulfide isomerase	ddpX	-2,64	--	D-alanyl-D-alanine dipeptidase
hslV	-2,36	--	ATP-dependent protease peptidase	ybiY	--	-2,24	Putative pyruvate formate-lyase 3 activating enzym
yraI	--	-2,34	Predicted periplasmic pilin chaperone	yfcS	--	-2,02	Putative fimbrial chaperone
hybD	--	-2,34	Hydrogenase 2 maturation endopeptidase	yhbU	--	-2,97	Putative collagenase
Protein synthesis							
rplNXEFRO-rpsNHE-rpmDJ	-7,96	2,01	50S ribosomal subunit protein; L14, L24, L5, L6, L18, L15, S14, S8, S5, L30, L36	rpsLG-fusA-tufA	-16,74	2,53	30S ribosomal subunit protein; S12, S7- Elongation factor G – Elongation factor Tu
rpsP-rimM-trmD- rplS	-15,96	2,72	30S ribosomal S16 protein - S16 rRNA-processing and G37 methyltransferase	rpsMKD-rplQ	-6,86	2.15	30S ribosomal subunit protein; S13. S11, S4 50S ribosomal L17
rpsJSCQrplCDW BVP-rpmC	-33,60	--	30S ribosomal subunit protein; S10, S19, S3, S17	rplKAJL	-20,49	--	50S ribosomal subunit protein; L11, L1, L10, L7
			50S ribosomal subunit protein; L3, L4, L23, L2, L22, L16, L29	rpsA	-8,56	--	30S ribosomal subunit protein; S1
rplU	-2,36	--	50S ribosomal subunit protein; L21, L27	rpsU	-7,42	--	30S ribosomal protein S21
rpsT	-3,85	--	30S ribosomal subunit protein; S20	rplM- rpsI	-9,12	--	50S ribosomal subunit protein; L13- 30S S9
rpsB- tsf	-9,47	--	30S ribosomal subunit protein; S2 - elongation factor EF-Ts	rpsFR-rplI	-24,38	--	30S ribosomal protein S6, S18- 50S L9
rplY	-7,87	--	50S ribosomal protein L25	rpmE	-8,98	--	50S ribosomal subunit protein L31
rpsO	-4,74	--	30S ribosomal protein S15	rpmE2	-2,02	--	50S ribosomal protein L31 type B
rpmF	-3,17	--	50S ribosomal protein L32	rumB	-3,22	--	23S rRNA m5U747 methyltransferase
rpmH	-10,56	--	50S ribosomal protein L34	rumA	-3,23	--	23S rRNA m5U1939 methyltransferase
rpmBG	-3,40	--	50S ribosomal protein L28, L33	metG	-2,92	--	Methionyl-tRNA synthetase

pcnB	-5,32	--	Poly(A) polymerase I	rluD	-3,36	--	23S rRNA pseudouridine synthase
pheST	-6,48	--	Phenylalanyl-tRNA synthetase α- and β-chain	lysS	-2,19	--	Lysine tRNA synthetase
alaS	-3,26	--	Alanyl-tRNA synthetase	tyrS	-2,81	--	Yrosyl-tRNA synthetase
spoT	-4,02	--	Bifunctional (p)ppGpp synthetase and hydrolase	prfA	-2,60	--	Peptide chain release factor RF-1
queA	-4,17	--	tRNA ribosyltransferase-isomerase	prfB	-3,94	--	Peptide chain release factor RF-2
relA	-3,02	--	GDP/GTP pyrophosphokinase	argS	-2,25	--	Arginyl-tRNA synthetase
yfiC	-2,39	--	tRNA m6A37 methyltransferase	infA	-2,75	--	protein chain initiation factor IF-1
proS	-2,03	--	Prolyl-tRNA synthetase	infB	-2,50	--	Protein chain initiation factor IF2
proQ	-4,19	--	RNA chaperone, involved in posttranscriptional control of ProP levels	rsmA	-2,86	--	16S rRNA dimethyladenosine transferase
truB	-3,07	--	tRNA pseudouridine synthase	hisS	-2,74	--	Histidyl-tRNA synthetase
leuS	-3,08	--	Leucyl-tRNA synthetase	cysS	-2,51	--	Cysteinyl-tRNA synthetase
aspS	-3,45	--	Aspartyl-tRNA synthetase	glyQ	-3,01	--	Glycine tRNA synthetase
Tgt	-6,06	--	tRNA-guanine transglycosylase	efp	-2,62	--	Elongation factor P
miaA	-2,97	--	tRNA(i6A37) synthase	rimI	-2,05	--	Acetylase for 30S ribosomal subunit protein S18
glnE	-2,05	--	Glutamine synthetase adenylyltransferase	asnC	-2,28	--	Asparaginyl-tRNA synthetase
pth	-2,49	--	Peptidyl-tRNA hydrolase	ycdL	--	-2,23	Predicted enzyme, isochorismatase homolog
yibK	--	-2,07	Putative tRNA/rRNA methyltransferase YibK	trmA	--	-2,17	tRNA (uracil-5-)-methyltransferase
yjcQ	--	-2,10	Multidrug efflux system protein MdtO	yehI	--	-2,63	Putative regulator
yhgE	--	-2,14	Putative transport protein	yghZ	--	-2,46	Aldo-keto reductase
thdF	--	-2,39	tRNA modification GTPase TrmE	Z4504	--	-2,22	Disrupted fimbrial protein
rutB	--	-2,23	isochorismatase family protein ycdL	Z3026	--	-2,19	Putative secreted protein
Z3058	--	-2,24	Putative OMP				
Amino acid biosynthesis							
trpLEDCB	-9,96	2,48	Tryptophan synthase operon	metE	-74,07	--	Cobalamin-independent homocysteine transmethylase
hisCBHA	-2,65	--	Biosynthesis of histidine	metH	-7,44	--	Cobalamin-dependent methionine synthase
leuABCD	-7,44	--	Isopropylmalate biosyntheses	cysE	-2,14	--	Serine acetyltransferase
thrABC	-4,67	--	Aspartokinase/homoserine dehydrogenase-kinase- threonine synthase	cysM	-6,75	--	Cysteine synthase
aroC-mepA-yfcL	-3,23	--	Chorismate synthase-murein endopeptidase	serB	-3,54	--	3-phosphoserine phosphatase
aroG	-4,27	--	2-dehydro-3-deoxyphosphoheptonate aldolases	asnA	-3,71	--	Asparagine synthetase
aroF	-12,85	--		asnB	-3,19	--	
ilvGMEDA	-10,59	--	Valine, leucine and isoleucine biosyntheses	aroKB	-4,34	--	Shikimate kinase- dehydroquinate synthase
ivbL-ilvBN	-2,64	--		aroL	-3,45	--	
ilvC	-49,34	--		lysC	-8,20	--	Aspartate kinase
hyuA	--	3,27	Phenylhydantoinase				
Fatty acid and phospholipids metabolism							

pssA	-2,77		Phosphatidylserine synthase	lgt	-2,13	--	Prolipoprotein diacylglyceryl transferase
plsC	-3,13		1-acylglycerol-3-phosphate O-acyltransferase	accA	-2,86	--	Acetyl-CoA carboxyltransferase
accBC	-4,91	--	Biotin carboxyl carrier protein / acetyl-CoA and biotin carboxylase	pldB	-2,40	--	Lysophospholipase L2
yceD-plsX-fabHDG	-5,33	2,11	Fatty acids biosynthesis	fabA	-3,42		3-hydroxydecanoyl-[acp] dehydrase
Z4853-Z4854	-2,17		Putative acyl carrier proteins	cdsA	-2,87	--	CDP-diglyceride synthase
Arp	--	2,62	Regulator of acetyl CoA synthetase				
Purines, pyrimidines, nucleosides, and nucleotides							
hflD-purB	-5,96		Lysogenization regulator- Adenylosuccinate lyase	gpt	-2,11	--	Xanthine-guanine phosphoribosyltransferase
purEK	-7,42		N5-carboxyaminoimidazole ribonucleotide mutase and synthetase	ndk	-4,44	--	Nucleoside di-P kinase
purHD	-4,99	--	AICARtransformylase/IMP cyclohydrolase-Phosphoribosylamine-glycine ligase	cmk	-6,28	--	Cytidylate kinase
purMN	-13,72	--	Phosphoribosyl aminoimidazole synthetase and glycinamide Formyltransferase	prsA	-2,39	--	Ribose-phosphate pyrophosphokinase
apt	-3,24	--	Adenine phosphoribosyltransferase	pyrF	-2,39	--	OMP decarboxylase
purC	-5,15	--	Phosphoribosylaminoimidazole-succinocarboxamide synthase	pyrG	-2,54	--	CTP synthetase
guaB	-4,16	--	IMP dehydrogenase	upp	-2,69	--	Uracil phosphoribosyltransferase
glmU	-5,01	--	N-acetylglucosamine-1-P uridyltransferase/glucosamine-1-P acetyltransferase	gsk	-2,31	--	Inosine-guanosine kinase
pyrD	-4,90	--	Dihydroorotate dehydrogenase	pyrC	-2,20	--	Dihydroorotase
purF	-9,94	--	Amidophosphoribosyl transferase	pyrH	-3,30	--	Uridylate kinase
trxB	-3,61	--	Thioredoxin reductase	tmk	-3,19	--	Thymidylate kinase
nrdA	-4,71	--	Ribonucleoside diphosphate reductase	adk	-2,29	--	Adenylate kinase
purT	-3,00	-2,17	Phosphoribosylglycinamide formyltransferase	purU	-2,85	--	Formyltetrahydrofolate deformylase
purL	3,84	--	Phosphoribosylformylglycinamide synthetase	udk	-2,06	--	Uridine/cytidine kinase
nrdG	--	-2,91	Anaerobic ribonucleotide reductase-activating protein	carAB	-9,95	--	Carbamoyl phosphate synthase
nrdF	--	-3,70	Ribonucleotide-di-P reductase subunit bet	gsk	--	-2,15	inosine-guanosine kinase
Biosynthesis of cofactors, prosthetic groups, and carriers							
pncB	-4,03	--	Nicotinate phosphoribosyltransferase	folE	-2,03	--	GTP cyclohydrolase I
bioFC	-5,44	--	Biotin biosynthesis , aminopelargonate biosynthesis	folK	-2,41	--	6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase
thiL	-3,26	--	Thiamin monophosphate kinase	folB	-2,15	--	Dihydroneopterin aldolase
entCE	-2,02	--	Isochorismate synthase EntC, MenF and enterobactin synthase subunit E	gshA	-2,52	--	Glutamate-cysteine ligase
menF	-3,38	--		ubiE	-2,11	--	Ubiquinone/menaquinone biosynthesis methyltransferase
hemC	-2,14	--	Hydroxymethylbilane synthase	pdxH	-2,08	--	Pyridoxamine 5'-phosphate oxidase
ribH	-2,53	--	Riboflavin synthase	ispU	-2,87	--	Undecaprenyl pyrophosphate synthase
dxs	-2,02	--	1-deoxy-D-xylulose-5-phosphate synthase	prmC	-2,41	--	Protein-(glutamine-N5) methyltransferase
yaeM	-2,07	--	1-deoxy-D-xylulose 5-phosphate reductoisomerase	nadR	-2,67	--	Nicotinamide-nucleotide adenylyltransferase
metF	-2,22	--	5,10-methylenetetrahydrofolate reductase	pabB	-2,27	--	Para-aminobenzoate synthase

ribF	-2,30	--	Bifunctional riboflavin kinase/FMN adenylyltransferase	grxC	-2,42	--	Reduced glutaredoxin 3
hemA	-2,31	--	Glutamyl-tRNA reductase	hemH	--	-2,69	Ferrochelatase
nadB	--	-2,53	L-aspartate oxidase	hemF	--	-2,11	Coproporphyrinogen III oxidase
Viral, prophage functions, mobile element functions (PAI)							
Z0955	-3,71	2,39	Unknown protein encoded by prophage CP-933K (OI #36)	Z1930	-2,55		Putative protease encoded within prophage CP-933X (OI #52)
Z1337	-2,23		Unknown protein encoded by cryptic prophage CP-933M (OI #44)	Z1643	-3,05		Hypothetical protein (OI #48)
Z5899-Z5901	-2,46		Putative ATP-dependent helicases (OI #172)	hfq	-2,34		HF-I, host factor for RNA phage Q beta replication
stx1AB	-4,14		Stx 1 subunit A and B encoded within prophage CP-933V (OI #93)	Z3943	--	-2,60	Putative enzyme; Integration, recombination (Prophage Related)
Z2039	--	-5,39	DicB, putative inhibitor of cell division encoded by cryptic prophage CP-933P	Z1852	--	-2,20	Putative holin protein of prophage CP-933C
ybiY	--	-2,24	Putative pyruvate formate-lyase 3 activating enzyme	nlp	--	-3,25	DNA-binding transcriptional regulator Nlp
Z3362	--	-2,45	Putative superinfection exclusion protein B of prophage CP-933V	yhbQ	--	-2.15	GIY-YIG nuclease superfamily protein
Non-coding RNA							
sraB	-2,87	--	Novel Novel sRNA, function unknown identified in a large scale screen	t44	-15,23	--	30S ribosomal protein S2
ryeE	--	-3,40	sRNA that promotes degradation of the ompX, yqaE, nadE, and luxS mRNAs	sraG	--	-3,83	PsrO small RNA
csrB	--	-3,60	sRNA inhibits CsrA-mediated acceleration of glg mRNA degradation	oxyS	--	-2,92	Oxidative stress regulator
micF	--	-2,44	Represses production of OmpF in response to environmental stress conditions	SibB	--	-2,09	sRNA (ryeD)

[a] : (--) represents the not-significant changes.

Table SIV. Pho-Box matrix

Pho-Box matrix based on 12 Pho-Boxes sequences from the known Pho genes in EDL933 identified by Yuan *et al*. 2006.

Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
phoB	C	T	G	T	C	A	T	A	A	A	T	C	T	G	A	C	G	C
pstS1	G	T	G	T	C	A	T	C	A	A	A	C	T	G	T	C	A	C
pstS2	C	T	G	T	C	A	C	A	T	T	C	C	T	T	A	C	A	T
pstS3	C	T	T	A	C	A	T	A	T	A	A	C	T	G	T	C	A	C
phoA1	C	T	G	T	C	A	T	A	A	A	G	T	T	G	T	C	A	C
phoA2	C	T	T	T	T	C	A	A	C	A	G	C	T	G	T	C	A	T
phoE1	C	T	G	T	A	A	T	A	T	A	T	C	T	T	T	A	A	C
phoE2	A	T	A	T	C	A	T	T	A	A	T	C	T	G	T	A	A	T
ugpB1	T	T	G	T	C	A	T	C	T	T	T	C	T	G	A	C	A	C
ugpB2	C	T	A	T	C	T	T	A	C	A	A	A	T	G	T	A	A	C
ugpB3	A	A	G	T	T	A	T	T	T	T	T	C	T	G	T	A	A	T
phnC	C	T	G	T	T	A	G	T	C	A	C	T	T	T	T	A	A	T
Base pairs Frequency																		
A	**16.7**	**8.3**	**16.7**	**8.3**	**8.3**	**83.4**	**8.3**	**58.3**	**33.3**	**75**	**25.0**	**8.3**	**0**	**0**	**25**	**41.7**	**91.7**	**0**
T	**8.3**	**91.7**	**16.7**	**91.7**	**25**	**8.3**	**75.1**	**25**	**41.7**	**25**	**41.6**	**16.7**	**100**	**25**	**75**	**0**	**0**	**41.7**
C	**66.7**	**0.0**	**0**	**0**	**66.7**	**8.3**	**8.3**	**16.7**	**25**	**0**	**16.7**	**75**	**0**	**0**	**0**	**58.3**	**0**	**58.3**
G	**8.3**	**0.0**	**66**	**0**	**0**	**0**	**8.3**	**0**	**0**	**0**	**16.7**	**0**	**0**	**75**	**0**	**0**	**8.3**	**0**

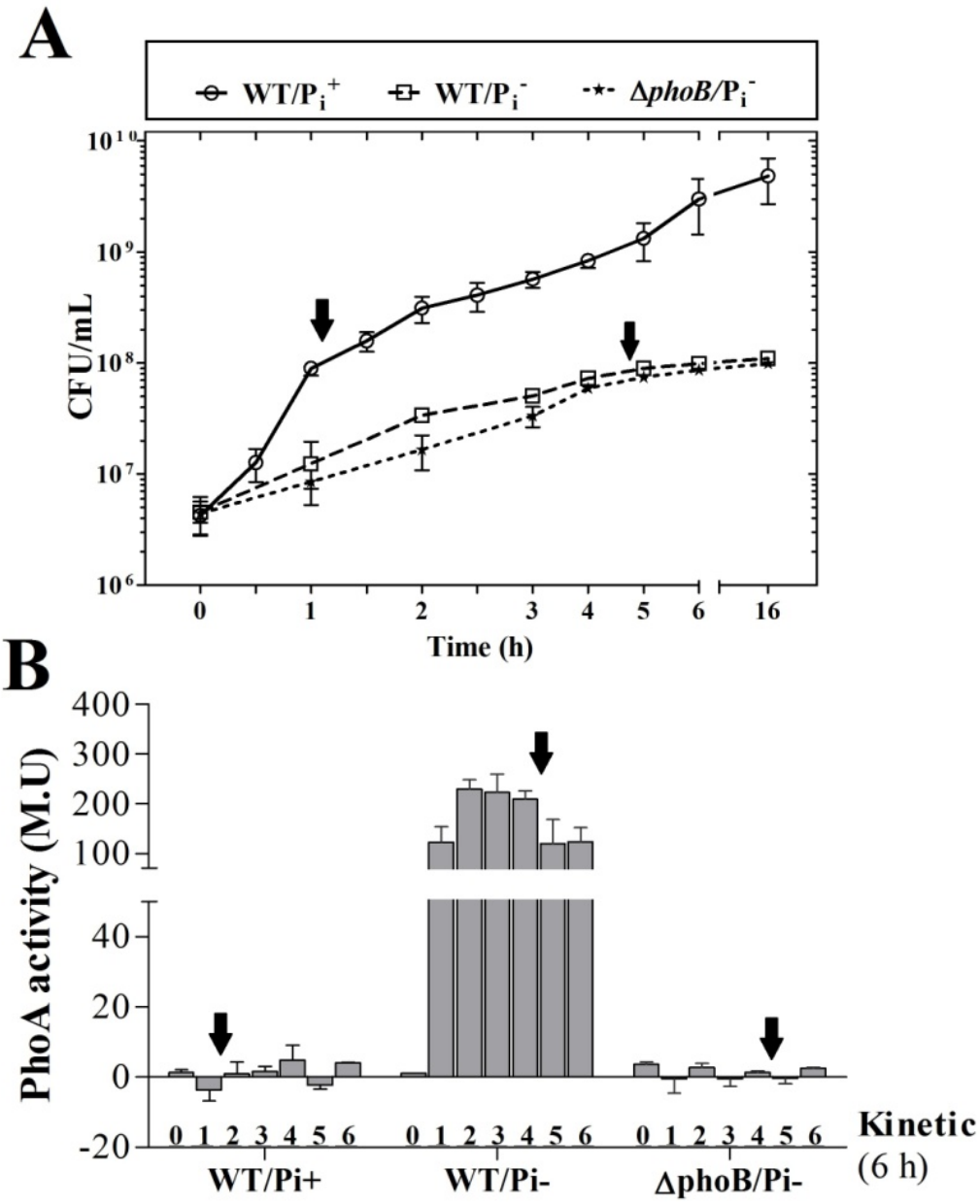
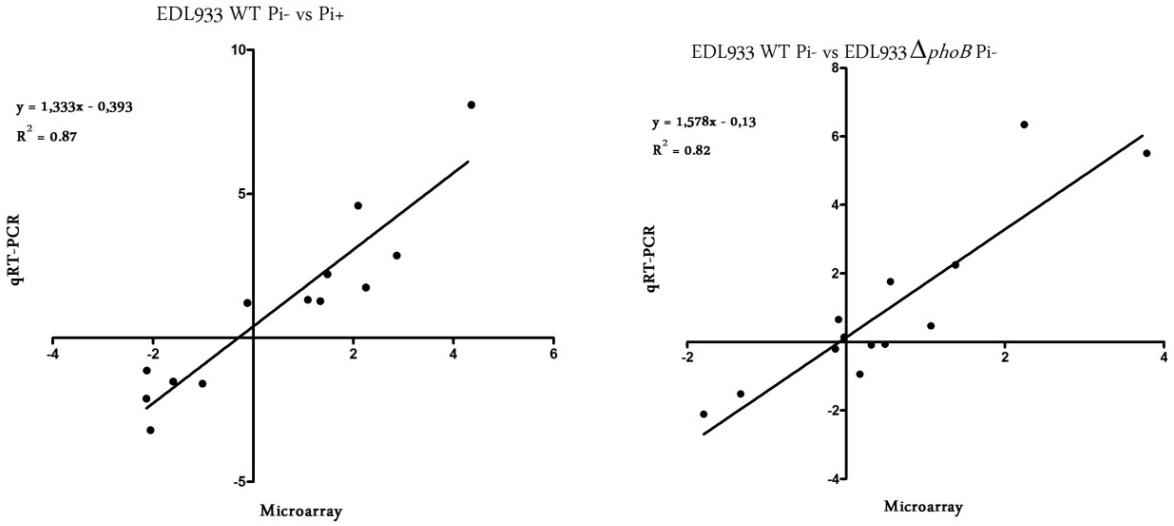


Figure S1. Growth and PhoA activity of WT strain and its isogenic mutant Δ*phoB*

A. Growth (CFU/mL) of EDL933 the WT strain and its isogenic mutants Δ*phoB* in MOPS medium supplemented with 1.32 mM (Pi+) or 1.0 µM (Pi-) KH_2PO_4 for 16 h at 37°C. **B.** PhoA activity monitoring during 6 h cultivation of EDL933 in Pi+ or Δ*phoB* in Pi- leads to abolition of the Pho regulon while it is activated in WT grown in Pi-. Arrows indicate the 2 comparisons points chosen for the rest of the experiments in this study.



	WT Pi-/WT Pi+ (log_2)		WT Pi-/ΔphoBPi+ (log_2)	
	µarray	qRT-PCR	µarray	qRT-PCR
phnL	4,36	8,09	2,24	6,35
phoB	2,10	4,59	3,79	5,51
ler	1,49	2,21	-0,02	0,14
espB	1,09	1,32	0,56	1,76
gadE	2,87	2,86	1,38	2,25
cro	2,25	1,75	-0,13	-0,21
stx2a	1,34	1,27	0,32	-0,09
cI	-1,01	-1,59	0,17	-0,94
lpxB	-2,12	-1,14	-0,09	0,65
recG	-1,60	-1,51	-1,79	-2,11
fhuA	-2,13	-2,11	-1,33	-1,52
nleG	-0,11	1,21	1,07	0,47
stx1a	-2,05	-3,21	0,49	-0,07

Figure S2. Microarry validation by qRT-PCR

Microarray results were validated by triplicate qRT-PCR on 13 representative genes. Each reaction was normalized to *tus* gene and the variation rate was calculated using $2^{-\Delta\Delta Ct}$ method.

ii. Article de recherche # 4. «Implication du régulon Pho dans la formation du biofilm et la mobilité des EHEC O157:H7».

Objectifs et approche utilisée

Cette étude avait pour objectif de déterminer le rôle du régulon Pho dans la formation de biofilm et la mobilité des EHEC O157 :H7. Nous avons caractérisé la formation de biofilm et la mobilité chez la souche EDL933 et ses mutants Δ*pst* et Δ*phoB*. La culture de la souche mutante EDL933 Δ*pst* dans un milieu minimal a pour conséquence un phénotype d'hyper biofilm et hypo motilité par rapport à la souche sauvage (WT). La comparaison transcriptome de mutant Δ*pst* à celui de WT en un milieu riche en Pi ainsi et la comparaison du transcriptome de WT en milieu pauvre en Pi à celui de WT en milieu riche en Pi nous a permis de sélectionner une liste de gènes potentiellement impliqués dans la formation de biofilm et motilité et qui sont différentiellement exprimés en réponse aux carences en Pi et/ou sous le contrôle du régulon Pho.

L'étude a été réalisée en utilisant des techniques de transcriptomique par hybridation sur puce d'ADN, culture bactérienne statique et tests classique de formation de biofilm et motilité chez *E. coli.* Des techniques de génétique bactérienne ont été utilisées pour créer les souches mutantes d'EDL933 et leurs compléments.

Contribution de l'étudiant

J'ai effectué la totalité des expérimentations. J'ai aussi écrit cet article qui présent ces résultats préliminaires qui vont être complétés par l'étudiant au PhD Philippe Vogeleer qui va poursuivre ce sujet en exploitant mes résultats et se chargera de soumettre cet article au journal Applied and Environmental Microbiology.

The Pho regulon involvement in EHEC O157:H7 biofilm formation and motility

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ABSTRACT

Under nutrient-deprived conditions, bacteria grow in a tightly packed community and encase themselves in a protective polysaccharide matrix, a process called biofilm formation. Such a state provides the bacteria with protection against physical stresses and allows slowed growth and metabolism, which extends bacterial survival. Although biofilm formation provides distinct advantages to food-borne bacteria, one such pathogen, enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is exposed to a wide range of nutritive stress, including Phosphate (Pi) limitation. Pi is commonly added to animal feeds and is highly available in human intestines. However, in open environments, EHEC responds to the phosphate (Pi) starvation by inducing the Pho regulon including the Pst system that sense Pi variation. In this study the constitutive expression of Pho regulon increased the biofilm amount formed by EHEC while its motility was decreased. The gene expression data suggested that Pi limitation and Pho regulon activation modulate the component biofilm matrix poly-β-1,6-N-acetyl-d-glucosamine (PGA). The results also indicate these conditions induce genetic pathways of curli and c-di-GMP production while the production pathway of indole was repressed. The preliminary results of this study highlighted the importance of extracellular (Pi) and intracellular (Pho) factors in biofilm formation of EHEC, which may impact its transmission and virulence.

Keywords. EHEC O157:H7, Pho regulon, Pst, Biofilm, motility, curli, indole, PGA.

Introduction

Escherichia coli O157:H7 is the predominant serotype of Enterohemorrhagic *E. coli*. (EHEC), the causative agent of severe gastrointestinal disease in humans and the majority of bloody diarrhea outbreaks in developed countries [603]. Although most cases of EHEC infection result in self-limiting resolution, 5 to 15% of cases in children develop an acute renal failure causing the hemolytic uremic syndrome (HUS), [604]. Healthy cows are the major reservoir of EHEC, which can persist in the farm environment, soil, water, and animal carcasses [19,605]. EHEC is an emerging food- and water-borne hazard because its transmission occurs by consumption of contaminated ground beef and raw milk and also from contaminated cattle through the fecal contamination of farm water and soil [312]. Also, increasing EHEC outbreaks were associated with produce, such as alfalfa sprouts, and apple cider [606]. Bacterial attachment is the first step in contamination of foods, and it is enhanced by producing biofilms [607]. *E. coli* O157 has shown the ability to attach, colonize, and form biofilms on a variety of biotic and abiotic surfaces [343,344,350,608]. These microbial communities in a matrix of exopolysaccharide (EPS) material produced by bacteria, including the EHEC, enhance their survival to various nutritional and oxidative stresses and their resistance to physical and chemical treatments [607,609,610]. Consequently, biofilms formed on food contact surfaces, can serve as continuous contamination sources of food spoilage bacteria and pathogens in the food processing environment [611]. EHEC are also able to form persistent biofilms on various green leaves and vegetables including spinach, lettuce, Chinese cabbage, celery, leeks, basil, and parsley [608,612]. The genetic mechanism of biofilm formation of *E. coli* O157:H7 is a complex process and is now beginning to be unveiled. The transcriptional regulator CsgD is central to biofilm formation, controlling the expression of the curli structural and export proteins, and the diguanylate cyclase, *adrA*, which indirectly activates cellulose production [344,349,350,613,614]. CsgD itself is highly regulated by two sigma factors (RpoS and RpoD), multiple DNA-binding proteins, silencing RNAs, and several GGDEF/EAL proteins acting through c-di-GMP [615]. However, Curli fibers and biofilm formation are poorly expressed in *E. coli* O157:H7 cultured under laboratory conditions in rich media. Recently, Uhlich *et al*., showed that EHEC biofilm could be blocked by two distinct mechanisms: i) insertion of DNA from a bacterial virus into the serotype O157:H7 biofilm-

regulating gene *mlrA* (*yehV*), causing its inactivation; and ii) the high occurrence of spontaneous DNA sequence-altering mutations in a second bioflm-regulating gene, *rpoS* [616]. Additionally, intercellular signal molecules, such as autoinducer-2 [354,355] and indole [347,617], are involved in biofilm formation of *E. coli* O157:H7.

In response to Pi starvation, we previously showed that EHEC differentially expressed many genes linked to biofilm formation and motility (Chekabab *et al*. in preparation). Therefore, the goal of this study was to determine the effect of Pho regulon induction on EHEC biofilm formation and motility.

Materials and methods

Bacterial strains, culture media and microarray experiment

The effect of Pho regulon on swimming motility and biofilm formation on EHEC O157:H7 was investigated using, strain EDL933 and its isogenic mutants Δ*pstCAB* (constitutive Pho) and Δ*phoB* (inactivated Pho) described elsewhere [460]. The WT and Δ*pst* strains from overnight LB cultures were inoculated and grown to mid log phase in 3-(Nmorpholino) propanesulfonic acid (MOPS Teknova®) minimal medium [573] supplemented with 0.2% (w/v) glucose, thiamine (0.1 µg/mL), and 1.32 mM of K_2HPO_4. Time-course monitoring of the Pho regulon expression was done by measuring the alkaline phosphatase (AP) activity as described previously [456,485].

Samples equal to $5x10^8$ CFU were taken from the mid log phase and processed for transcriptomes analysis. Ten µg of RNAs were extracted and retro-transcribed. One µg of the fragmented and biotinylated cDNAs was hybridized onto Affymetrix GeneChip® *E. coli*. The data were processed using FlexArray® software using Robust Multichip Average (RMA) normalization procedure. The levels of transcription obtained from 3 biological replicates of each experimental condition were compared using the EB (Wright & Simon) algorithm. A comparison was conducted between the Δ*pst* mutant and the WT strain. The differential expression conditions corresponded to 2-fold change (FC) cut-off and p-value < 0.05.

Static biofilm assay

E. coli O157:H7 EDL933 strains from overnight culture in LB broth were inoculated (1:100) into M9 minimal medium (+0.2% glycerol) and then a second overnight incubation at 37 °C. From this, 150 µl of 1:100 dilutions were dispensed into wells of 96-well polyvinyl chloride flexible microplates (BD Flacon). After 30 h incubation at 30 °C, the plates were rinsed twice in deionized water to remove loosely attached bacteria. Each well was stained with 150 µl of 0.01% crystal violet for 20 min at room temperature. The staining solution was removed, and wells were rinsed in deionized water two times. After drying, the crystal violet bound to the biofilm was solubilized with 150 µl of ethanol: acetone (80:20) mixture. The absorbance was measured at 595 nm and the means were calculated from 3 plates representing 3 biological experiments and 4 technical repetitions. Four non-inoculated wells were used as negative controls and served as the blank measure. Biofilm formation of EDL933 strains was also assessed in the MOPS medium in similar culture conditions mentioned above for the microarray experiment.

Motility assay

EDL933 strains were platted on LB agar and grown overnight at 37 °C. Individual colonies were collected with sterile toothpicks and stabbed into agar plates containing 20 mL motility medium BBL™ [Beef Extract0.3%; Pancreatic Digest of Gelatin 1%; NaCl 0.5%; Agar 0.4%) auditioned with 1 % Triphenyltetrazolium Chloride (TTC)]. Plates were incubated for 8 h at 37 °C and the diameter of the diffuse growth zone was measured. *E. coli* ATCC® 25922 and *Staphylococcus aureus* ATCC® 25923 were included respectively as motility positive and negative controls.

Results

Phenotypic characterization of Pho-dependent biofilm and motility in EDL933

Because the Pho regulon is known to modulate swimming motility and biofilm formation in various bacterial species [433,448-450], the impact of *phoB* inactivation and Pho constitutive expression (Δ*pst*) on these two phenotypes was further examined.

For biofilm phenotype characterization, bacteria cells were grown in minimal medium M9 or MOPS (supplemented with % glycerol) in static cultures for 30 h at 30C. These conditions have been developed for reproducibility of the results and measurement of biofilm by the standard crystal violet method.

As shown in Figure 1, inactivation of *pst* resulted in a 3.6-fold increase in biofilm formation in M9 medium (Δ*pst* OD_{595}= 1.37±0.01; wild type OD_{595}= 0.38±0.03). The complemented strain carrying the *pst* operon *in trans* showed partial phenotypic complementation (OD_{595}= 0.81±0.11) corresponding to 1.7-fold decrease compared to Δ*pst*. Inversely the deletion of *phoB* showed a 1.7-fold decrease in biofilm formation (OD_{595}= 0.22 ±0.014), that was fully complemented to wild type level in the mutant strain carrying the *phoBR* operon *in trans*. Similar significant differences were observed in MOPS medium in which all strains formed slightly less biofilm than in M9 (Figure 1C).

As shown in Figure 2, inactivation of *pst* resulted in a significant decrease (~2-fold Δ*pst* 9.5 ±0.87 mm; wild type 18.5 ±1.2 mm) in the swimming zone in the semi-solid agar (a commercial BBL medium rich in Pi) whereas no difference was observed in the Δ*phoB* strain. In this motility phenotype, the complementation of Δ*pst* was not conclusive. However, in the Δ*phoB* complemented strain, motility was slightly decreased compared to the wild type strain.

These results indicate that Pho regulon expression could be implicated in the phenotypic fitness of EHEC by controlling the switch between the planktonic and sedentary lifestyles. This was demonstrated for several species including *V. cholerae* and *P. aeruginosa* where PhoB was described as hypo biofilm and hyper motility regulator [433,448-450]. In this study, opposite effects were observed; The Pho regulon expression was correlated with over production of biofilm and hypo motility phenotypes.

Transcriptomic analysis revealed Pi and Pho regulon modulation of biofilm and motility genes expression.

In our previous work we observed some biofilm and motility-related genes that were differentially expressed in response to Pi limitation and/or *phoB* deletion (Chekabab et al. 2014 in press). In this study the transcriptome of EDL933 in response to Pho constitutive expression (Δ*pst*) was analyzed. To do this, the Affymetrix *E. coli* genome microarray was used for the hybridization of cDNA retro-transcribed from RNA isolated from Δ*pst* grown in

MOPS high Pi. The data were compared to the wild type strain and resulted in 205 up regulated genes (Table II) and 473 down regulated genes (table III). The differentially expressed genes, in response to Δ*pst* deletion, were assigned to the Cluster of Orthologous Gene (COG) classification (Figure 3).

Upregulated genes included those encoding proteins of unknown function, transport and binding proteins, energy and central intermediary metabolism, and transcription. Genes that were downregulated also included the unknown function genes, as well as those involved with protein fate; protein synthesis; DNA metabolism; purine, pyrimidine, nucleoside and nucleotide pathways; and cell envelope proteins. A large number of genes involved in stress responses and hypothetical proteins were also differentially expressed as well as some virulence genes including LEE genes. The expression patterns of the genome-wide transcriptional response observed, shared similarities with transcriptomic analyses of *E. coli* K-12 during phosphate starvation as well as pathogen avian *E. coli* (APEC) and *Citrobacter rodentium* Δ*pst* mutants [456,459].

Validation of microarray results was achieved using qRT-PCR. A comparison of the expression of 11 genes by microarray hybridizations and qRT-PCR demonstrated a high level of concordance between the datasets, which is represented by a correlation coefficient of 0.92 (Figure 4).

In table I, a list of 48 identified genes related to biofilm formation were differentially expressed in response to Pi (WT Pi- versus WT Pi+) and/or to Δ*pst* (Δ*pst* Pi+ versus WT Pi+). This genes list included 3 groups showed in the Venn diagram (Figure 5).

Group 1 includes 19 genes with expression levels that changed in the WT strain in response to Pi limitation. Six up regulated genes are involved in the curli and colanic acid biosynthesis and one gene encodes a c-di-GMP cyclase (*ycdT*). Thirteen downregulated genes were mainly involved in the tryptophan metabolism and transport, the LPS biosynthesis, a putative fimbrial precursor (*z2200*) and a c-di-GMP phsophodiesterase (*yciR*).

Group 2 includes 19 genes showing expression modulated in response to both Pi limitation and Δ*pst* mutation. Interestingly, in this group, the gene encoding the biofilm regulator BssR was highly expressed. However, the operon encoding PGA was downregulated in the Δ*pst* mutant while it was upregulated in response to low Pi.

Group 3 included 10 genes having an expression level affected in response to Δ*pst* mutation and not to Pi limitation. Nine upregulated genes encode for putative fimbrial proteins, a regulator of cellulose synthase (*bcsB*), and diguanylate cyclases (*ydeH, yeaI*). The *wzx* gene encoding antigen flippase, involved in lipopolysaccharides (LPS) biosynthesis, was down regulated in this group.

Discussion

This study showed the decreased motility and increased biofilm formation by EDL933 expressing the Pho regulon. Furthermore, a list was drawn of differential biofilm gene expression in conditions of Pi starvation and when the Pst system is abolished, and that both activate the Pho regulon.

In this study, the genes expression involved in flagella metabolism and regulation were not changed. However, we previous found that the absence of PhoB increased the expression of genes involved in regulation of flagella biosynthesis (Chekabab et al. 2014 in press). This could be correlated to the decreased motility of the Δ*pst* mutant (expressing constitutively the Pho regulon).

In response to Pi limitation, and independently of Pho regulon activation, the biofilm genotype showed up regulation of genes involved in curli production and a strong gene repression of tryptophan metabolism and indole synthesis. This is consistent with a previous report demonstrating that strong biofilm formation by EDL933 depends on its ability to produce curli and repress indole signaling [348]. The curli is a thin coiled protein structure on the surface of cells reported to bind with components in the eukaryotic extracellular matrix [618]. Indole is known as a metabolite of amino acid tryptophan, which has been proved to inhibit biofilm formation by *E. coli* O157:H7 [347,619]. However, in response to Pho regulon induction, and independently of Pi starvation, up regulation involved genes encoding cellulose and c-di-GMP biosynthesis while LPS biosynthesis gene *wzx* was repressed. Cellulose is one of key components of the biofilm matrix, while LPS may not accumulate significantly in the matrix [620]. Furthermore, it is known that cellulose production is directly stimulated by the curli transcriptional regulator CsgD and both curli and cellulose play a synergistic role in biofilm formation [349,621]. Moreover, the c-di-GMP is known as a biofilm inducer by

stimulating the biosynthesis of various adhesins and exopolysaccharides and inhibits bacterial mobility by controlling the switch between planktonic and sedentary lifestyles [424]. In this study, the gene *ycdT* implicated in c-di-GMP biosynthesis was induced in response to Pi. This is corroborated with the recent report demonstrating increased *E. coli* O157:H7 biofilm formation in the absence of a specific c-di-GMP phsophodiesterase [622]. The β-1,6-N-acetyl-D-glucosamine polymer (PGA) is an important component of the biofilm matrix of *E. coli* [620] and promotes attachment to solid surfaces, cell–cell adherence, and stabilization of biofilm structure [623,624]. In this study, the *pga* operon was induced in response to low Pi while it was repressed in the Δ*pst* mutant. This discrepancy could be explained by the level of Pho regulon induction. Indeed, the PhoA activity, reporting level of Pho regulon activation, was much higher in the Δ*pst* mutant than that of wild type in a Pi limiting condition (data not shown).

The pO157 plasmid encodes several genes known to modulate EDL933 biofilm formation [190] and the pO157-cured strain showed a biofilm defect [625]. However, because the Affymetrix GeneChip® *E. coli* does not include probes for the plasmid genes, the role of the plasmid was not assessed in this study. Nevertheless, many biofilm-related components, such as curli, indole, and PGA and c-di-GMP biosynthesis are modulated in response to Pi limitation and/or the induction of the Pho regulon. To complete this study, work is underway using reporter fusion to determine the effect of Pho regulon on those biofilm components. Moreover, the Δ*pst* mutant as a hypo-motile and hyper-biofilm former is an ideal candidate for random mutagenesis in order to characterize the potential mutants that will restore the biofilm and/or motility to the wild type levels.

This study suggests that *E. coli* O157:H7, by forming biofilms in environments where Pi is limited, could pose significant risk to continuous contamination. Thereby, for managing the risk of transmission, it would be interesting to monitor the level of Pi in water and food additives, and to determine the threshold of Pi concentration below which *E. coli* O157: H7 form biofilm in foods and in food preparation steps.

Figures and tables

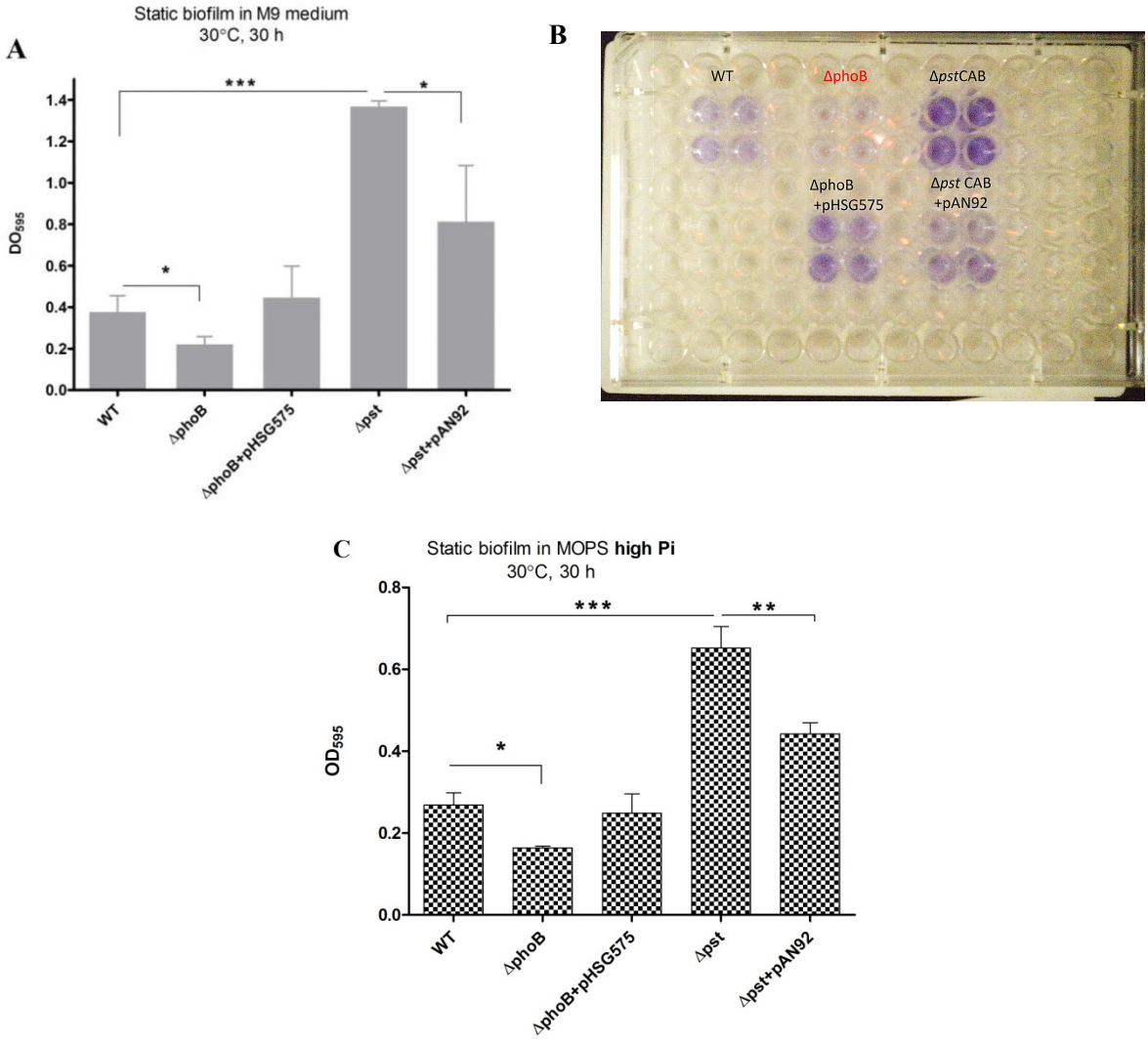


Figure 1. Biofilm formation by EDL933 wild type strain and its mutant's Δ*pst* and Δ*phoB*.

Biofilm formed in M9 medium (**A** and **B**) and in MOPS high Pi (**C**) after 30 h at 30°C. Error bars represent the standard deviation (n=3). The asterisk symbols indicating the significant differences with *p* value <0.05 *; <0.01**, and <0.001***.

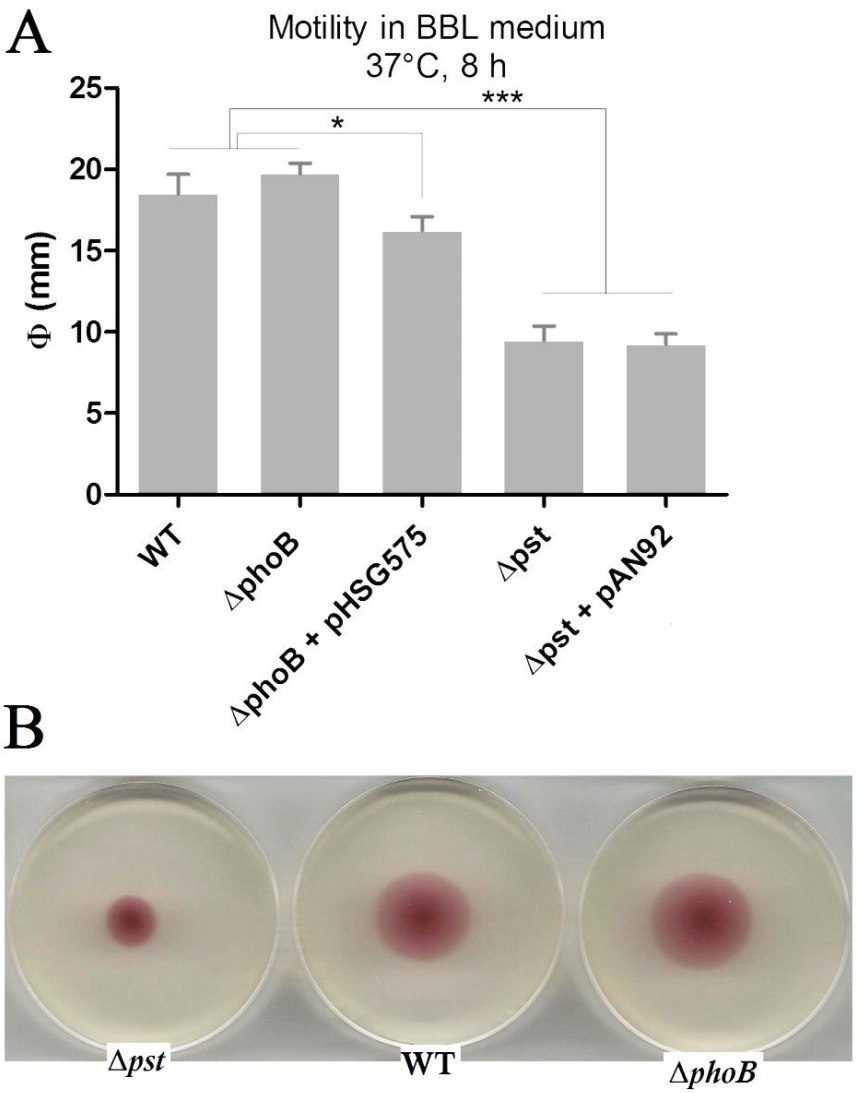


Figure 2. Swimming motility by EDL933 wild type strain and its mutant's Δ*pst* and Δ*phoB*.

The motility determined in BBL medium after 8 h at 37°C. The diameter indicated in A was measured from the diffuse growth zone (Red halo in B). Error bars represent the standard deviation (n=3). The asterisk symbols indicating the significantly differences with *p* value <0.05 * and <0.001***.

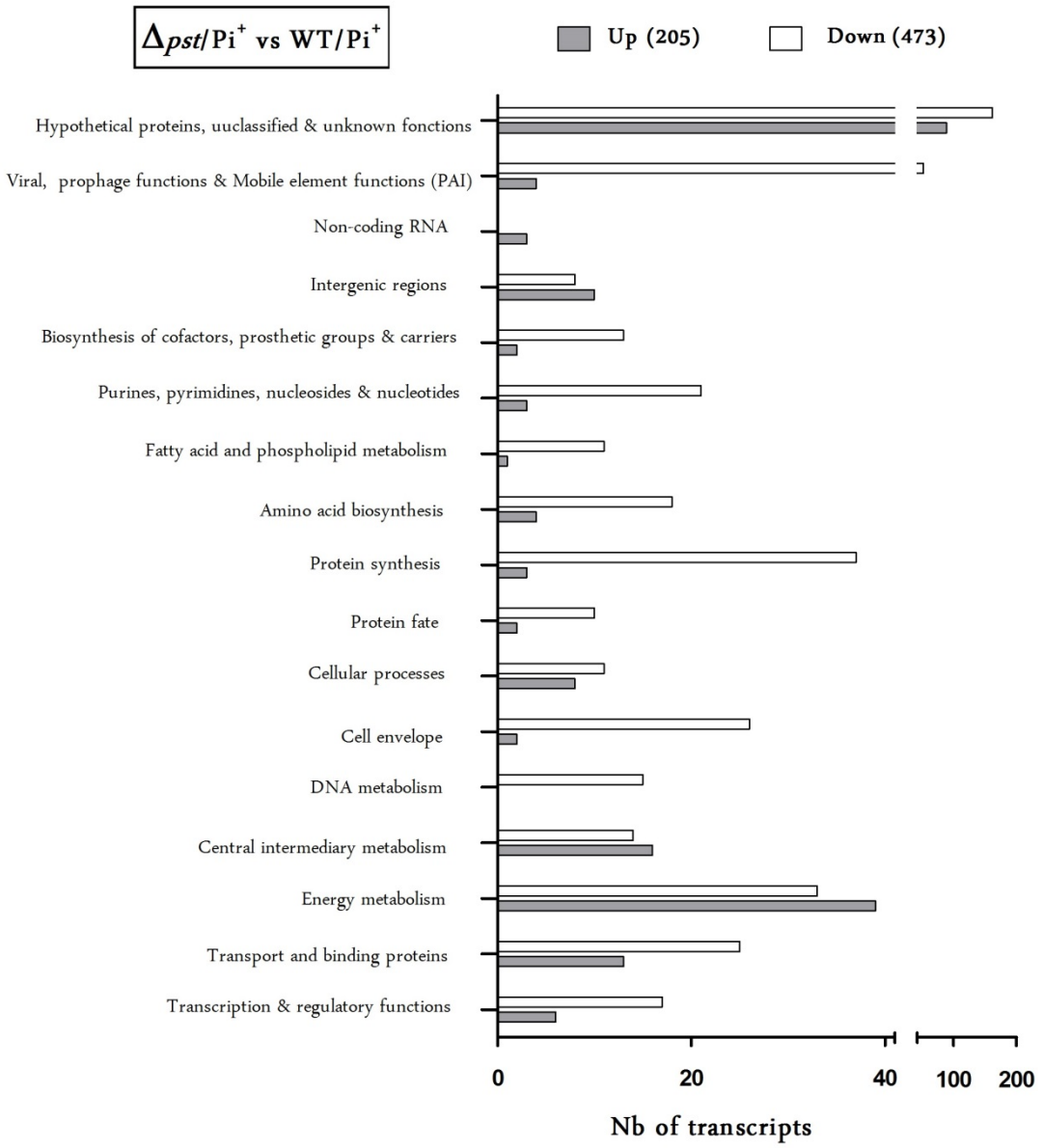
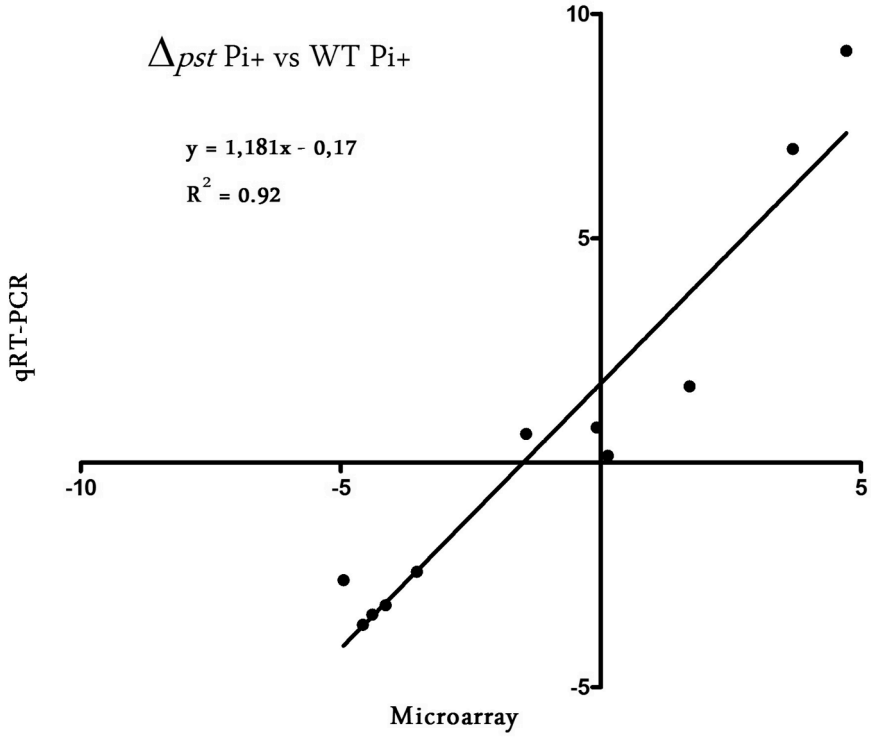


Figure 3. Functional classification of microarray results.

Classification of microarray results according to TIGR's Comprehensive Microbial Resource (CMR) showing the up and down-regulated genes in Δ*pst* compared to the WT strain. The y-axis represents the numbers of differentially transcripts according to the functional class.



	Δ*pst* P_i+ vs WT P_i+ (log_2)	
	µarray	qRT-PCR
ler	-4.14	-3.17
espB	-4.39	-3.38
sepZ	-4.57	-3.61
escV	-3.53	-2.43
tir	-4.95	-2.61
gadE	1.27	1.70
stx2A	-0.08	0.78
stx2B	0.14	0.15
phnL	4.72	9.17
phoB	3.69	6.99
lpxB	-1.44	0.65

Figure 4. Microarray results were validated by triplicate qRT-PCR on 11 representative genes. Each reaction was normalized to *tus* gene and the variation rate was calculated using $2^{-\Delta\Delta Ct}$ method

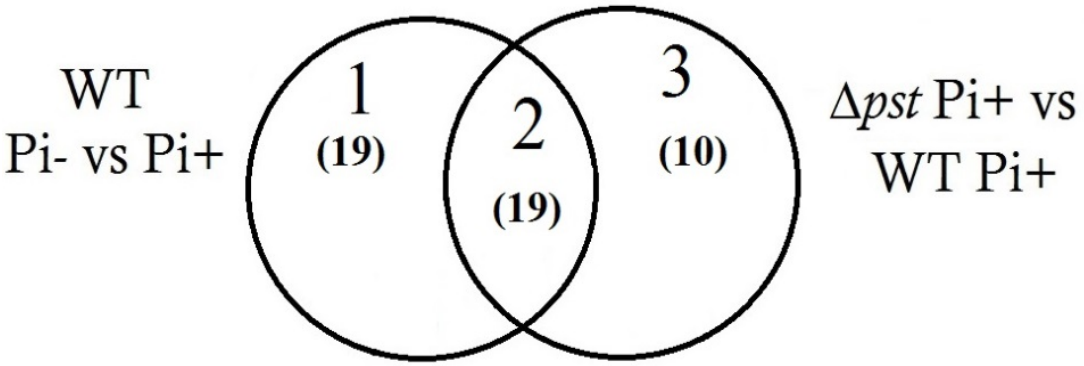


Figure 5. Venn diagram showing genes implicated in EDL933 biofilm formation that are differentially expressed in response to Pi limitation and/or in Δ*pst* mutant

Table I. Genes implicated in EDL933 biofilm formation that are differentially expressed in response to Pi limitation and/or in Δ*pst* mutant

Gene / ORF	Function and description	µArray Fold Change		Group
		Pi	Δ*pst*	
Fimbriae, pili & curli				
yagZ_b0293	Cryptic Mat fimbrillin gene	**2,60**	**-5,72**	2
b1505	Outer membrane usher protein fimD precursor	**-2,47**	**-2.01**	2
fimZ_b0535	Predicted DNA-binding transcriptional regulator FimZ	1.64	**2.67**	3
Z2200	Putative type 1 fimbrial protein precursor / putative major fimbrial subunit F9	**-3,28**	-1.50	1
Z2201	Chaperone protein fimC precursor	**-3,02**	**-2,17**	2
Z0024	Putative type-1 fimbrial protein	**2,32**	**2.50**	2
yehD_Z3279	Putative fimbrial-like protein	1.54	**2,21**	3
Z5220	Putative fimbrial protein	1.45	**2,17**	3
Z4968	PapC-like porin protein involved in fimbrial biogenesis	1.14	**2,07**	3
Z3276	Putative fimbrial protein	1.20	**2,00**	3
csgB_b1041	Curlin nucleator protein, minor subunit in curli complex	**2,07**	**2.48**	2
csgD_b1040	DNA-binding transcriptional regulator CsgD activator for csgBA	**2,20**	1.53	1
csgE_b1039	Curli assembly protein CsgE /curli production assembly/transport componen	**2,53**	1.32	1
csgF_b1038	Curli assembly protein CsgF	**2.47**	1.38	1
PGA				
pgaA_b1024	Biofilm adhesin polysaccharide PGA export, predicted OM protein	**4,57**	**-4,52**	2
pgaB_b1023	Adhesin polysaccharide PGA export lipoprotein polysaccharide deacetylase activity needed for export	**6,74**	**-4,30**	2
pgaC_b1022	N-glycosyltransferase	**6,32**	**-4,50**	2
pgaD_b1021	Adhesin polysaccharide PGA synthesis required for biofilm	**8,43**	**-2.01**	2
Lipopolysaccharide				
lpxD_b0179	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	**-2,73**	**-2,24**	2
lpxA_b0181	UDP-N-acetylglucosamine acyltransferase	**-2,23**	-1.42	1
lpxB_b0182	Lipid-A-disaccharide synthase	**-4,36**	**-2,71**	2
rlpB_Z0788	LPS-assembly lipoprotein RlpB	**-4,07**	**-2,44**	2
fcl_Z3197	Fucose synthetase	**-2,22**	-1.73	1
wzx_Z3201	O antigen flippase Wzx	1.1	**-2,93**	3
waaP_Z5054	Lipopolysaccharide core biosynthesis protein RfaP	**-2,09**	**-2,01**	2
waaL_Z5049	Lipid A-core : Surface polymer ligase and putative LPS biosynthesis enzyme	**-2,81**	-1.88	1
waaQ_Z5056	Lipopolysaccharide core biosynthesis protein	**-2,91**	**-2,72**	2
Tryptophan metabolism				
trpB_b1261	Tryptophan synthase, beta subunit	**-3,63**	1.47	1
trpC_b1262	Indole-3-glycerolphosphate synthetase	**-5,26**	1.35	1
trpD_Z2548	Anthranilate phosphoribosyltransferase	**-9,19**	1.16	1
trpE1_Z2546	Anthranilate synthase component I	**-7,12**	-1.32	1
trpE2_Z2547	Anthranilate synthase component I	**-9,96**	-1.18	1
rpL_b1265	trp operon leader peptide	**-9,94**	-1.03	1
mtr_b3161	Tryptophan permease /tryptophan transporter of high affinity	**-2,78**	1.42	1
Other biofilm-related genes				
bssS_b1060	Biofilm formation regulatory protein BssS /// biofilm regulator	**3,44**	**3,12**	2
bssR_b0836	Biofilm regulatory prot BssR /repressor of biofilm by indole transport regulation	**13,51**	**18,58**	2
b3023_Z4375	Hypothetical protein / transcriptional repressor for mcbR biofilm gene	**2,88**	**3,01**	2
wcaJ_b2047	Predected colanic acid biosynthesis/ putative UDP-glucose lipid carrier transferase	**2,04**	1.96	1
bcsB_yhjN	Regulator of cellulose synthase, cyclic di-GMP binding	1.69	**2.35**	3
ycdT_b1025	Diguanylate cyclase	**2,09**	-1.29	1
ydeH_b1535	Diguanylate cyclase, required for pgaD induction	1.27	**3.06**	3
yeaI_Z2825	Predicted diguanylate cyclase	1.33	**3.94**	3
yhjN_b3532	Regulator of cellulose synthase, cyclic di-GMP binding	1.69	**2.35**	3
yciR_b1285	Cyclic-di-GMP phosphodiesterase; csgD regulator; modulator of Rnase II stability	**-2,32**	-1.58	1
yrfG_b3399	Putative phosphatase /GMP/IMP nucleotidase	**-2,78**	-1.79	1
guaA_c3026	GMP synthetase (glutamine aminotransferase)	**-2,63**	**-2,30**	2
Z1528	rtn-like protein	**2.13**	1.28	1

Table II. Functional classification of upregulated genes in EDL933 Δ*pst* mutant

Gene name/ ORF	Function and description	µArray Fold Change
Transcription & regulatory functions 6		
phoB-R	DNA-binding response regulator in TCS with PhoR (or CreC)	12.93
yieN	regulatory ATPase RavA b3746/ yieN	2.12
CusR	DNA-binding response regulator in TCS with CusS	2.60
TtdR	transcriptional activator of ttdABT	3.20
ECs1199	putative antirepressor protein	3.16
Transport & binding 13		
pstS	High-affinity phosphate periplasmic substrate-binding protein	84.40
phoE	Outer membrane phosphoporin protein	48.61
ugpBAE	Glycerol-3-phosphate transporter	42.99
napA	Nitrate reductase catalytic subunit	28.48
CusB	copper/silver efflux system membrane fusion protein	4.27
fruA	PTS system fructose-specific transporter subunits IIBC	5.36
ycgC	dihydroxyacetone-specific PTS enzyme	2.49
feoA	ferrous iron transport protein	3.33
malE	maltose ABC transporter periplasmic protein	2.51
nikA	periplasmic binding protein for nickel	3.62
Z4271	ABC transporter ATP-binding protein	3.23
c3509	ABC transporter ATP-binding protein	3.60
Energy metabolism 39		
frdABCD	Fumarate reductase	6.93
napFDAGHBC	nitrate reductase pathway	7.08
nrfABCD	nitrite reductase formate-dependent	8.78
focA-pflBA	pyruvate-formate metabolism	4.91
nirBDC	nitrite reductase pathway	38.28
hypBDE	hydrogenases enzymes	2.74
dmsAB	dimethyl sulfoxide reductase	6.67
fruK	fructose-1-phosphate kinase	4.79
pfkA	6-phosphofructokinase	3.85
tpiA	triosephosphate isomerase	2.78
pykA	pyruvate kinase	2.09
yiaI	hydrogenase, 4Fe-4S ferredoxin-type component	3.28
yhjA	cytochrome C peroxidase	3.00
yliI	soluble aldose sugar dehydrogenase	2.09
cydB	cytochrome D ubiquinol oxidase subunit II	2.55
ansB	periplasmic L-asparaginase II	2.32
qor	quinone oxidoreductase, NADPH-dependent	2.03
pgk	phosphoglycerate kinase	3.19
malP	maltodextrin phosphorylase	3.22
b1525	succinate semialdehyde dehydrogenase	2.63
Central intermediary metabolism 16		
phnCDEF-HIJKLMNO	Phosphonate transporter and metabolisme	90.80
phoA- psiF	Alkaline phosphatase/P-starvation-inducible prot	119.59
amn	AMP nucleosidase	7.29
gadhE	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	7.74
ackA	acetate kinase A and propionate kinase 2	3.17
Cell envelope 2		
yciD	outer membrane protein W	4.29
cusC	copper/silver efflux system outer membrane protein	7.42
Cellular processes 8		
ahpCF	Alkyl hydroperoxide reductase subunit C	2,69
uspA	Universal stress protein A , global response regulator	3.17
vgrE	Rhs element protein (OI #65)	3.23
yiiT	universal stress protein UspD	4.31
yedW	response regulator in TCS with YedV	2.22
sodB	superoxide dismutase	2.53

katG	peroxidase/catalase HPI	3.14
Protein fate 2		
yhbU	putative collagenase	5.11
pepT	peptidase T	4.52
Protein synthesis 3		
yibD	putative glycosyl transferase	45.86
miaA	isopentenylpyrophosphate tRNA-adenosine transferase	2.42
Z1972	predicted adhesin	4.65
Amino acid biosynthesis 4		
hisD	Histidinol dehydrogenase	4.12
hisC	histidinol-phosphate aminotransferase	4.20
hisG	ATP phosphoribosyltransferase	4.16
hisB	histidinol-phosphatase/imidazoleglycerol-phosphate dehydratase	2.93
Fatty acid and phospholipids metabolism 1		
cdh	CDP-diacylglycerol pyrophosphatase	4.46
Purines, pyrimidines, nucleosides, and nucleotides 3		
nrdD	anaerobic ribonucleoside triphosphate reductase	4.46
nrdG	anaerobic ribonucleotide reductase-activating protein	2.72
udp	uridine phosphorylase	2.65
Biosynthesis of cofactors, prosthetic groups, and carriers 2		
moaE	Molybdopterin guanine dinucleotide biosynthesis protein	2,53
cysG	siroheme synthase	2.65
Viral, prophage functions, Mobile element functions (PAI) 4		
nohB	putative DNA packaging protein of prophage CP-933X (OI#52)	4.89
nlp	DNA-binding transcriptional regulator Nlp /	2.47
Z2122	putative holin protein of prophage CP-933O (OI#57)	3.88
Z0321	putative AraC-type regulatory protein encoded in prophage CP-933H	5.36
ncRNA 3		
csrB_b4408	ncRNA	2.87
gcvB_b4443		2.64
Z5401		2.60

Table III. Functional classification of downregulated genes in EDL933Δ*pst* mutant

Gene name/ ORF	Function and description	µArray Fold Change
Transcription & regulatory functions 17		
rpoD	DNA-directed RNA polymerase subunits	-2.42
purR	DNA-binding transcriptional repressor	-3.55
rnb	exoribonuclease I	-4.00
rnpA	ribonuclease P	-4.77
rnhB	ribonuclease HII, degrades RNA of DNA-RNA hybrids	-3.22
rbfA	ribosome-binding factor A	-2.07
yehU	predicted sensory kinase in TCS with YehT	-2.05
icc	adenosine monophosphate phosphodiesterase	-2.06
era	GTP-binding protein Era	-2.17
basS	sensory histidine kinase in TCS with BasR	-2.18
emrR	transcriptional repressor of microcin B17 synthesis and multidrug efflux	-2.44
betI	DNA-binding transcriptional repressor	-3.04
glnL	nitrogen regulation protein NR(II)	-2.97
ycdQ	N-glycosyltransferase	-4.50
ECs2027	putative transcriptional regulator LYSR-type	-2.73
c3307	RNA polymerase, sigma S (sigma 38) factor	-4.80
ECs1941	putative transcriptional regulator	-5.74
Transport & binding 25		
cysPUWA	Sulfate / thiosulfate ABC transporter	-8.23
potAB	Putrescine / spermidine ABC transporter	-3.16
btuB	Vitamin B12/cobalamin outer membrane transporter	-3.34
lysP	Lysine transporter	-2.49
gltJ	Glutamate and aspartate transporter	-2.49
trkH	K+ transporter	-2.34
acrB	acriflavin resistance protein / multidrug efflux system	-2.00
sapA	antimicrobial peptide transport ABC transporter periplasmic binding protein	-2.18
msbA	lipid transporter ATP-binding/permease protein	-2.24
yjjK	putative ABC transporter ATP-binding protein	-2.55
putP	major sodium/proline symporter	-2.68
metI	DL-methionine transporter permease subunit	-2.29
uup	ABC transporter ATPase component	-2.52
metN	DL-methionine transporter ATP-binding subunit	-2.59
yddA	putative ATP-binding component of a transport system	-3.94
livH	leucine/isoleucine/valine transporter subunit	-2.82
b1729	part of a kinase /// putative symporter ydjN	-3.99
Z0463	putative response regulator; hexosephosphate transport	-2.66
hisQMP	Histidine ABC transport	-3.17
Energy metabolism 33		
cyoABCDE	Cytochrome o ubiquinol oxidase – heme O synthase	-9.02
sucAB	2-oxoglutarate dehydrogenase, thiamin-requiring and dihydrolipoamide succinyltransferase	-2.86
sdhCDAB	Succinate dehydrogenases	-20.30
gcvTP	Glycine cleavage system	-3.48
glpEG	Thiosulfate sulfurtransferase and intramembrane serine protease	-2.38
glgXC	Glycogen debranching enzyme and glucose-1-phosphate adenylyltransferase	-2.27
fdoG	Formate dehydrogenase	-3.34
lpdA	Lipoamide dehydrogenase	-2.79
fdx	Reduced ferredoxin, electron carrer protein	-2.98
gltA	Type II citrate synthase	-3.19
ppsA	phosphoenolpyruvate synthase	-3.12
yccY	phosphotyrosine-protein phosphatase	-3.87
speA	arginine decarboxylase	-2.30
pntB	pyridine nucleotide transhydrogenase	-2.36
sfcA	malate dehydrogenase	-2.67
acs	acetyl-CoA synthetase	-2.56
ilvA	threonine dehydratase	-2.63
pntA	NAD(P) transhydrogenase	-2.36

udhA	soluble pyridine nucleotide transhydrogenas	-2.60
gpsA	glycerol-3-phosphate dehydrogenase (NAD+)	-2.22
ybiK	Isoaspartyl peptidase	-2.20
atpB	F0F1 ATP synthase subunit A	-2.17
Central intermediary metabolism 14		
ureDABCEF	ureases accessory and structural proteins	-3.18
cysDNC	Sulfate adenylyltransferases (cysDN) - adenylylsulfate kinase (cysC)	-10.21
cysJI	Sulfite reductase	-15.05
codA	Cytosine deaminase	-2.98
b2529	electron transport complex protein required for the reduction of SoxR	-3.07
b1631	electron transport complex protein RnfG required for the reduction of SoxR	-2.61
DNA metabolism 15		
holC	DNA polymerase III subunit chi	-2.94
holB	DNA polymerase III subunit delta'	-2.65
holA	DNA polymerase III subunit delta	-2.83
dnaB	replicative DNA helicase	-2.25
dnaE	DNA polymerase III subunit alpha	-2.20
dnaG	DNA primase	-2.63
fis	Fis DNA-binding transcriptional dual regulator	-4.80
recF	recombination protein F	-3.55
mutY	adenine DNA glycosylase	-3.17
metK	methionine adenosyltransferase	-3.28
dinG	ATP-dependent DNA helicase	-2.20
b3289	16S rRNA methyltransferase	-2.14
b4389	DNA repair protein RadA	-2.62
b1633	endonuclease III	-3.01
Z1800	hypothetical protein predicted in DNA metabolism	-4.05
Cell envelope 26		
rlpA	septal ring protein, suppressor of prc, minor lipoprotein	-2.44
rlpB	LPS-assembly lipoprotein	-2.62
mreCD	Peptidoglycan biosynthesis	-3.23
murFGC	Peptidoglycan biosynthesis	-2.78
bamC	OMP Assembly Complex	-3.93
bamA	OMP Assembly Complex	-3.35
nlpD	activator of AmiC murein hydrolase activity, lipoprotein	-5.92
mrdAB	Cell shape; peptidoglycan synthetase; penicillin-binding protein 2	-2.62
mepA	Murein DD-endopeptidase	-3.27
wecBC	UDP-N-acetylglucosamine 2-epimerase; UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase	-2.24
pal	peptidoglycan-associated outer membrane lipoprotein	-3.10
mrcA	peptidoglycan synthetase	-2.91
lnt	apolipoprotein N-acyltransferase	-3.05
wzx	O antigen flippase Wzx	-2.93
mltC	membrane-bound lytic murein transglycosylase C	-3.00
mraY	phospho-N-acetylmuramoyl-pentapeptide transferase	-2.31
lpxB	lipid-A-disaccharide synthase	-2.71
waaQ	LPS core biosynthesis protein	-2.72
ycaH	lipid A 4'kinase	-2.23
ddlB	D-alanine--D-alanine ligase	-2.07
Z2201	Chaperone protein fimC precursor	-2.17
Cellular processes 11		
tolQRA	Tol-Pal Cell Envelope Complex membrane spanning protein in TolA-TolQ-TolR complex	-3.70
ybeX	Magnesium and cobalt efflux protein corC	-3.21
ybhC	Acyl-CoA thioesterase, lipoprotein	-2.58
ftsW	Essential cell division proteins	-2.38
cvpA	Colicin V production protein	-10.55
surE	Stationary phase survival protein SurE	-3.14
betA	Choline dehydrogenase	-2.86
ftsK	DNA translocase FtsK	-2.38
ECs2198	MokW toxin production and resistance	-23.28
Protein fate 10		
prlA	preprotein translocase subunit SecY	-4.28
yebU	rRNA (cytosine-C(5)-)-methyltransferase RsmF	-3.05

surA	Peptidyl-prolyl cis-trans isomerase	-3.30
prfC	Peptide chain release factor 3	-2.92
pepA	Leucyl aminopeptidase	-3.25
prc	carboxy-terminal protease	-2.66
secB	Preprotein translocase subunit	-2.58
pcm	Protein-L-isoaspartate O-methyltransferase	-2.20
b0955	Putative ATP-dependent protease	-2.23
b1488	D-ala-D-ala dipeptidase, Zn-dependent	-3.50
Protein synthesis 37		
rplNXEF- rpsNHE	50S ribosomal subunit protein; L14, L24, L5, S14, S8, S5	-2.54
rpsP- rplS	30S ribosomal S16 protein, 50S ribosomal subunit protein L19	-4.77
rpsLG	30S ribosomal subunit protein; S12, S7	-3.67
rpsJ-rplCDWB	30S ribosomal subunit protein; S10, 50S ribosomal subunit protein; L3, L4, L23, L2	-4.63
rplKA	50S ribosomal subunit protein; L11, L1	-3.94
rpsU	30S ribosomal protein S21	-3.62
rpsA	30S ribosomal subunit protein; S1	-3.05
rpsF	30S ribosomal subunit protein S6	-2.96
spoT	Bifunctional (p)ppGpp synthetase and hydrolase	-2.94
relA	GDP/GTP pyrophosphokinase	-2.38
pheS	Phenylalanyl-tRNA synthetase α-	-3.60
ksgA	dimethyladenosine transferase	-2.95
valS	valyl-tRNA synthetase	-3.29
spoU	tRNA guanosine-2'-O-methyltransferase	-2.17
tgt	Queuine tRNA-ribosyltransferase	-2.01
proQ	RNA chaperone, involved in posttranscriptional control of ProP levels	-2.48
infB	Protein chain initiation factor IF2	-2.44
queA	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	-2.54
rpmH	50S ribosomal subunit protein L34	-2.71
prfB	Peptide chain release factor RF-2	-2.66
sfhB	23S rRNA pseudouridine synthase D	-2.44
guaA	GMP synthetase (glutamine aminotransferase)	-2.30
Z4858	putative enzyme	-2.82
ECs3063	elongation factor P	-2.03
Amino acid biosynthesis 18		
leuABC	Isopropylmalate biosyntheses	-4.31
thrABC	Aspartokinase/homoserine dehydrogenase-kinase- threonine synthase	-5.70
ilvGD	Valine, leucine and isoleucine biosyntheses	-4.67
ilvC	Valine, leucine and isoleucine biosyntheses	-2.16
cysM	Cysteine synthase	-5.01
aroF	2-dehydro-3-deoxyphosphoheptonate aldolases	-3.97
metE	Cobalamin-independent homocysteine transmethylase	-2.36
serA	D-3-phosphoglycerate dehydrogenase	-3.40
serB	3-phosphoserine phosphatase	-2.96
ilvB	acetolactate synthase catalytic subunit	-2.54
lysA	diaminopimelate decarboxylase, PLP-binding	-2.37
serC	phosphoserine aminotransferase	-2.37
cysK	cysteine synthase A, O-acetylserine sulfhydrolase A subunit	-2.56
Fatty acid and phospholipids metabolism 11		
accA	Acetyl-CoA carboxyltransferase	-2.56
accBC	Biotin carboxyl carrier protein / acetyl-CoA and biotin carboxylase	-2.32
fabA	3-hydroxydecanoyl-[acp] dehydrase	-2.69
fabH	AcetylCoA ACP transacylase	-3.84
fadD	Long-chain-fatty-acid--CoA ligase	-2.12
cdsA	CDP-diglyceride synthase	-2.47
Z4853-Z4854	Putative acyl carrier protein	-2.24
Purines, pyrimidines, nucleosides, and nucleotides 21		
purB	Adenylosuccinate lyase	-6.24
purEK	N5-carboxyaminoimidazole ribonucleotide mutase and synthetase	-7.28
purHD	AICARtransformylase/IMP cyclohydrolase-Phosphoribosylamine-glycine ligase	-4.93
purMN	Phosphoribosyl aminoimidazole synthetase and glycinamide Formyltransferase	-12.06
purC	Phosphoribosylaminoimidazole-succinocarboxamide synthase	-7.11
purF	Amidophosphoribosyl transferase	-8.52

pyrCD	Dihydroorotases	-2.13
pyrH	Uridylate kinase	-3.05
pyrF	OMP decarboxylase	-2.39
tmk	Thymidylate kinase	-2.31
prsA	Ribose-phosphate pyrophosphokinase	-2.56
trxB	Thioredoxin reductase	-3.17
carAB	Carbamoyl phosphate synthase	-6.43
ndk	Nucleoside di-P kinase	-5.19
guaB	IMP dehydrogenase	-4.37
cmk	cytidylate kinase	-3.05
Biosynthesis of cofactors, prosthetic groups, and carriers 13		
bioAFCD	Biotin biosynthesis , aminopelargonate biosynthesis	-4.41
iscS	Cysteine desulfurase	-3.40
folC	Bifunctional folylpolyglutamate synthase/ dihydrofolate synthase	-2.06
hemK	N5-glutamine methyltransferase, modifies release factors RF-1 and RF-2	-2.25
pabB	Para-aminobenzoate synthase component I	-2.23
nadR	Nicotinamide-nucleotide adenylyltransferase	-2.37
entE	Enterobactin synthase subunit E	-2.44
dxs	1-deoxy-D-xylulose-5-phosphate synthase	-2.39
ubiC	Chorismate pyruvate lyase	-2.38
pdxA	4-hydroxy-L-threonine phosphate dehydrogenase	-3.25
Viral, prophage functions, Mobile element functions (PAI) 52		
Ler-escRST	Lee-encoded regulator (LEE1 operon) Oi#148	-17.59
grlR	negative regulator of the ler gene	-11.92
grlA	Positive regulator of the ler gene	-17.80
sepZ-escIJCD	(LEE2 operon) Oi#148	-23.83
escVN-sepQH	(LEE3 operon) Oi#148	-11.59
cesF	T3SS chaperone	-7.36
map	T3SS secreted effector	-23.65
tir-cesT-eae	Translocated intimine receptor, chaperone, adhesine(LEE3 operon) Oi#148	-30.83
escD	T3SS structure protein	-12.14
sepL-espADBD2F-escF	Switching protein EspL, translocators EspAD, effector EspB and EspF, structural EscF (LEE4 operon)	-17.92
Z2400	Partial putative represssor protein encoded within prophage CP-933R	-8.32
Z2104	Putative ARAC-type regulatory protein of CP-933O (OI#57)	-10.67
ECs2209	Putative repressor protein encoded within prophage CP-933O (OI#57)	-9.82
Z2090	Putative repressor protein encoded within prophage CP-933O (OI#57)	-25.43
Z2108	Phage protein YjhS encoded within prophage CP-933O (OI#57)	-3.27
Z1878	Putative Bor protein precursor of bacteriophage BP-933W (OI#45)	-5.35
lomW (Z1489)	Putative outer membrane protein Lom precursor of bacteriophage BP-933W (OI#45)	-4.56
Z1930	Putative protease encoded within prophage CP-933X (OI#52)	-4.72
Z3314	Putative tail component of prophage CP-933V (OI#93)	-2.96
stx1A (Z3344)	Shiga-like toxin 1 subunit A encoded within prophage CP-933V (OI#93)	-2.64
Z1377	Putative tail component encoded by cryptic prophage CP-933M/ CP-933R/ CP-933V	-2.40
Z0955	Putative outer membrane protein encoded in CP-933K (Oi#36)	-25.43
Z4330	Putative transposase (OI#122)	-3.57
Z1150	Pseudo /// unknown in IS (OI#43)	-2.33
ECs1332	Putative colicin immunity protein	-3.04

Discussion générale

Depuis leur première description en 1982, les EHEC ont émergé comme pathogènes d'origine alimentaire et hydrique causant chez les humains des diarrhées, des colites hémorragiques et le syndrome SHU. Durant la dernière décennie, on a constaté une hausse du nombre d'éclosions des EHEC O157:H7 associées aux contaminations environnementales de l'eau et par le biais de produits frais tels que les feuilles de salade ou les légumes. Ceci rend encore plus difficile la détection de ces pathogènes dans l'eau et la surveillance de la qualité microbiologique et l'innocuité de la nourriture. En effet, les EHEC ont développé des comportements et stratégies leur permettant de persister dans l'environnement, et de ce fait constituent encore un risque menaçant la santé publique.

A. Les protozoaires : réservoir possible des EHEC dans l'environnement

Ce projet de thèse investigue la survie des EHEC en association avec *A. castellanii*. Au bout de 3 semaines de culture, le nombre de bactéries EHEC est significativement plus élevé en présence de l'amibe qu'en son absence. Cette présence d'amibes permet aussi à la bactérie de rester viable et cultivable et de moins entrer en état VBNC. Le régulon Pho est requis pour la cultivablité des EHEC en présence de l'amibe, alors que les Stx n'ont pas d'implication apparente dans cette association à long-terme. Par ailleurs, les observations microscopiques pendant les premières 24h de co-culture montrent que les EHEC se situent dans les phagosomes à l'intérieur des amibes, et cette survie intracellulaire est plus importante en absence des Stx. En revanche la présence des EHEC entraine un taux basal de toxicité et mortalité des amibes, plus important qu'en présence de d'*E.coli* non-pathogène. Cette mortalité est plus accentuée en présence des Stx qu'en leur absence. Ainsi, l'association EHEC/amibe semble possible et bénéfique pour la bactérie, du moment que la présence de l'amibe lui permette de prolonger sa survie et l'intégrité de son pouvoir infectieux. De plus, les Stx semblent contrôler partialement cette interaction hôte/pathogène pendant le premier jour de leur association.

B. Les Stx: un moyen de défense des EHEC contre les cellules eucaryotes

Chez les EHEC, les gènes des Stx sont portés par des bactériophages lysogéniques ou tempérés. Par ailleurs, ces phages se retrouvent souvent en liberté dans l'environnement. En effet, avec une population estimée à plus de 10^{30} dans le monde, ces bactériophages sont parmi les entités biologiques les plus abondantes sur terre [626]. Des études récentes indiquent qu'une large portion sont des phages tempérés [627]. Ces phages peuvent insérer leurs génomes dans celui de leur hôte bactérienne et former des lysogènes. Les gènes exprimés à partir des prophages intégrés peuvent modifier le phénotype de l'hôte lors du processus de conversion lysogénique, donnant ainsi de nouvelles caractéristiques à l'hôte [160]. Beaucoup de bactériophages tempérés codent pour des exotoxines dont les Stx, les toxines cholérique et botulique, qui sont connues pour être agents causatifs de maladies humaines. Dépendamment du phage, ces exotoxines sont exprimées durant l'un des cycles lysogène ou lytique et parfois les deux. Dans les cas du phage encodant les Stx, la production de toxines requiert la phase lytique du phage alors qu'elle est réprimée durant le cycle lysogénique. Donc la production de Stx se produit en parallèle avec le cycle lytique et la lyse bactérienne [176,628]. Dans d'autres cas, tels que la production de la toxine diphtérique par *Corynebacterium diphtheriae*, la production et la sécrétion de toxines sont indépendantes de la réplication du phage où elle est encodée [629,630].

Les bactéries ont évolué de nombreuses méthodes pour repousser les prédateurs. La diffusion coordonnée d'exotoxines codées par des phages peut signifier une stratégie possible de défense anti-prédateur [387]. Les bactériophages codant des toxines sont très fréquemment isolés à partir de milieux où leurs cibles mammifères présumées ne sont pas répandues [631], suggérant que ces toxines serviraient comme défense anti-prédateur. Dans ce contexte, il a été montré que la production de Stx2 réduit la population du prédateur *T. thermophila*, conférant ainsi un avantage de survie à la bactérie qui porte ce phage codant cette toxine [388].

Les bactériophages codant des toxines tuent les cellules eucaryotes en inactivant des facteurs et/ou des voies universellement conservées chez les organismes eucaryotes. L'effet cytotoxique de ces toxines requiert leur préalable entrée dans le cytoplasme de la cellule cible et ensuite leur clivage par des enzymes intracellulaire de la cellule hôte. Souvent, l'accès au cytoplasme des cellules de mammifères requiert une liaison de la toxine à des récepteurs

membranaires spécifiques, suivie de son endocytose médiée par ces récepteurs [632-634]. Les eucaryotes unicellulaires prédateurs de bactéries ne sont pas supposés contenir de tels récepteurs, et/ou leur capacité d'endocytose médiée par ces récepteurs n'est pas établie. C'est pour cette raison que les mécanismes précis par lesquels les exotoxines pénètrent dans le cytoplasme de ces cellules sont largement inconnus. Récemment il a été montré que l'intoxication de *Tetrahymena* par Stx est facilitée par une capture médiée par un supposé récepteur glycoconjugé qui est distinct du récepteur Gb3 de mammifère [635]. Mis à part l'endocytose médiée par des récepteurs, les molécules de toxines peuvent également accéder au cytoplasme de la cellule phagocytaire protozoaire ciblée via une libération à partir des bactéries déjà ingérées [635]. Néanmoins, nos résultats montrant l'implication des Stx dans le taux de survie intra-amibe et dans la mortalité des amibes soulignent l'intérêt d'utiliser ces amibes comme modèle d'interaction hôte/pathogène pour étudier la pathogénicité des EHEC.

C. La carence en Pi et l'induction du régulon Pho entraînent un stress général chez les EHEC et modulent leur virulence

En utilisant conjointement les systèmes PhoB-R et Pst, les EHEC sont capables de s'adapter au manque de Pi en rétablissant un équilibre physiologique face à ce stress nutritif. Dans ce projet de thèse, nous avons utilisé des conditions de laboratoires contrôlées, avec un milieu de culture minimal pauvre en Pi, mimant le stress nutritif en Pi rencontré par les EHEC dans l'environnement aquatique et parfois *in vivo* dans l'intestin de l'hôte. Dans ces conditions, la souche EHEC EDL933 modifie son expression génique pour s'adapter à la carence en Pi et de manière concomitante des changements dans l'expression des gènes de virulence surviennent. En effet, dans des conditions limitées en Pi, les changements transcriptionnels géniques des EHEC sont associés à l'acquisition et au métabolisme de Pi, et également avec des mécanismes de réponses au stress général, y compris le stress oxydatif et les gènes de virulence.

a. Stress généralisé en réponse à la carence en Pi

Globalement, le profil transcriptionnel de la souche EHEC EDL933 cultivée dans un milieu pauvre en Pi indique que, en plus d'augmenter l'expression des gènes associés aux voies d'acquisition et de conservation de Pi, de nombreux gènes associés directement avec le stress bactérien global sont aussi différentiellement exprimés. De nombreux éléments suggèrent que le régulon Pho et la réponse au stress sont liés. En effet, les régulons Pho et RpoS sont des réseaux de régulation interdépendants pendant les réponses adaptatives bactériennes (Fig. 1) [338]. RpoS est un facteur sigma impliqué dans la réponse cellulaire à de nombreux stress. Il est également impliqué dans la régulation génétique durant la phase stationnaire et aussi dans des milieux nutritifs limités (Figs. 1 et 2) [581].

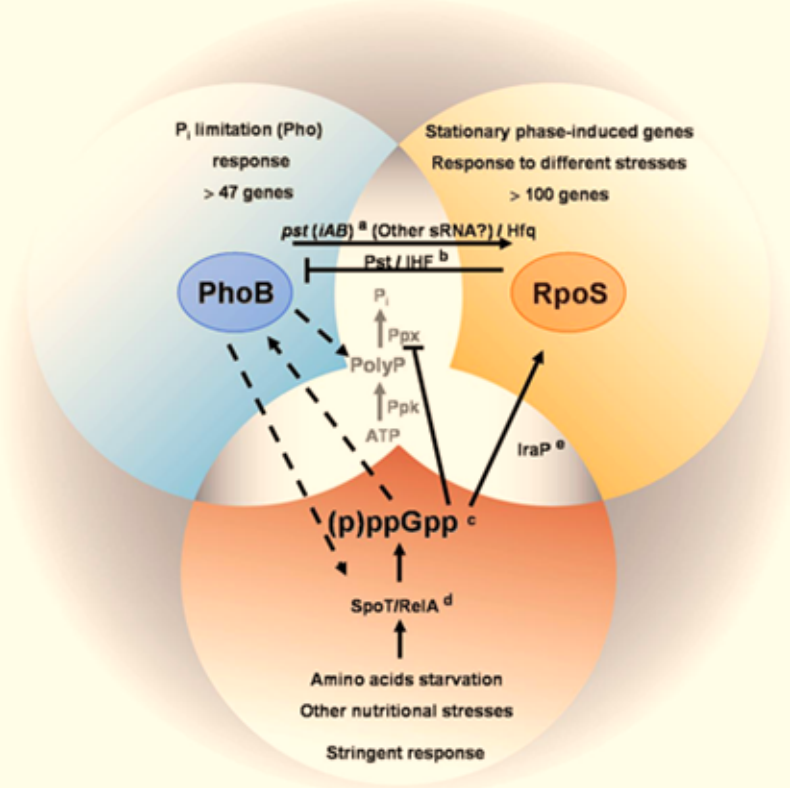


Figure 1. Schéma illustrant les interactions entre le régulon Pho, la voie de biosynthèse des Poly P, RpoS et la réponse stringente (Lamarche et al. 2008) [338].

Les lignes en discontinu représentent des effets qui pourraient être soit direct ou indirect. RpoS contribue à réprimer le régulon Pho en stimulant directement l'expression de l'opéron *pst* de manière IHF-dépendante [636-638]. Un niveau cellulaire basal de ppGpp est essentiel pour l'expression adéquate du régulon Pho. De plus, l'induction du régulon Pho est observée de façon concomitante avec la synthèse ppGpp, jusqu'à ce qu'un certain seuil est atteint, c'est à dire une concentration intracellulaire ppGpp qui induit une inhibition de la synthèse protéique [639,640].

La carence en Pi induit une accumulation du ppGpp SpoT-dépendante. Cela favorise la transcription du gène codant pour l'«antiadaptor» IraP qui interfère avec la prestation de RpoS à la protéase ClpXP en bloquant l'action de RssB, une protéine adaptatrice pour la dégradation des RpoS [583,641].

Le gène *rpoS* est exprimé sous différentes conditions de croissance, mais la régulation par RpoS et sa production sont largement tributaires de la stabilité post-transcriptionnelle (Fig. 2) [581,582]. Cependant, dans nos conditions de culture de la souche EDL933 dans un milieu pauvre en Pi, le gène *rpoS* n'est pas différentiellement exprimé. Néanmoins, dans cette condition, le gène *iraP* est induit. IraP code pour un anti-adaptateur qui améliore la stabilité et l'accumulation de RpoS, en inhibant son adressage au dégradosome ClpXP (Fig. 1) [583,584]. Ces résultats suggèrent donc une augmentation de la stabilité du facteur RpoS chez les EHEC cultivées en milieu pauvre en Pi, conduisant à l'expression de nombreux gènes de régulation RpoS-dépendants.

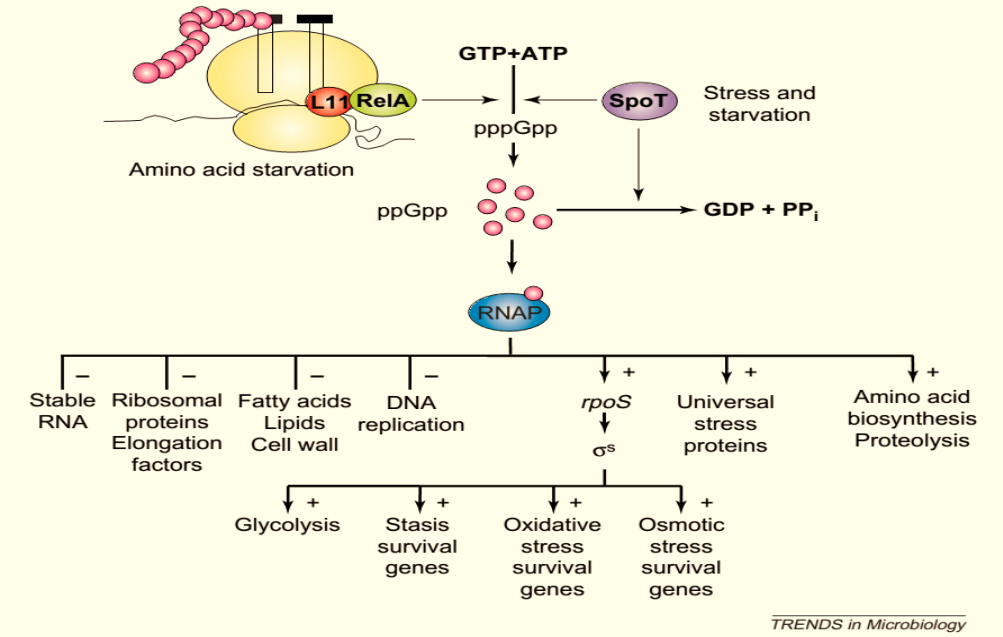


Figure 2. Schéma de la réponse stringente (Magnusson et al. 2005) [642].

En réponse aux carences et stress, le pppGpp est produit par deux voies parallèles à partir d'ATP et de GTP, ensuite converti en ppGpp. Le ppGpp se lie RNAP et redirige la transcription prioritairement vers les gènes de résistance aux stress et survie à la famine, plutôt que les gènes liés à la croissance.

Nos résultats d'analyses transcriptomiques montrent que l'expression du régulon Pho lors du stress nutritif de Pi entraine également une diminution de biosynthèse d'ARN

ribosomal et de production de ribosomes. Ceci est typique de la réponse stringente pendant la famine en acides aminés [643,644], mais aussi en réponse à différentes carences nutritionnelles ou à des circonstance causant l'arrêt de croissance [645]. En effet, le croisement ou «inter-régulation» par les 3 voies (régulons Pho/RpoS et réponse stringente) a été précédemment démontrée (Fig. 2). La molécule effectrice contrôlant le modulon stringence est le ppGpp, qui est produit chez *E. coli* par les 2 voies: RelA et SpoT-dépendantes. Dans nos comparaisons transcriptomiques chez la souche EHEC EDL933, les gènes codant RelA et SpoT sont différentiellement exprimés.

En outre, certains des gènes impliqués dans les réponses aux stresses oxydatif et acide ont été différentiellement exprimés en réponse à la limitation en Pi et en comparaison avec le mutant Δ*phoB*. Ces réponses aux stress ont été également observées dans le mutant Δ*pst* chez des souches d'APEC et de *C. rodentium* [456,459].

b. Influence de la carence en Pi et du régulateur PhoB sur la virulence des EHEC

En utilisant l'analyse transcriptionnelle, le western-blot et un test ELISA, nous avons montré que la carence en Pi active l'expression et la production de gènes de virulence chez les EHEC: LEE et la toxine Stx2. La mutation Δ*phoB* affecte l'expression de certains de ces gènes de virulence. Cela indique que la virulence des EHEC est partiellement modulée par PhoB, ou les gènes régulés par PhoB (Fig. 3).

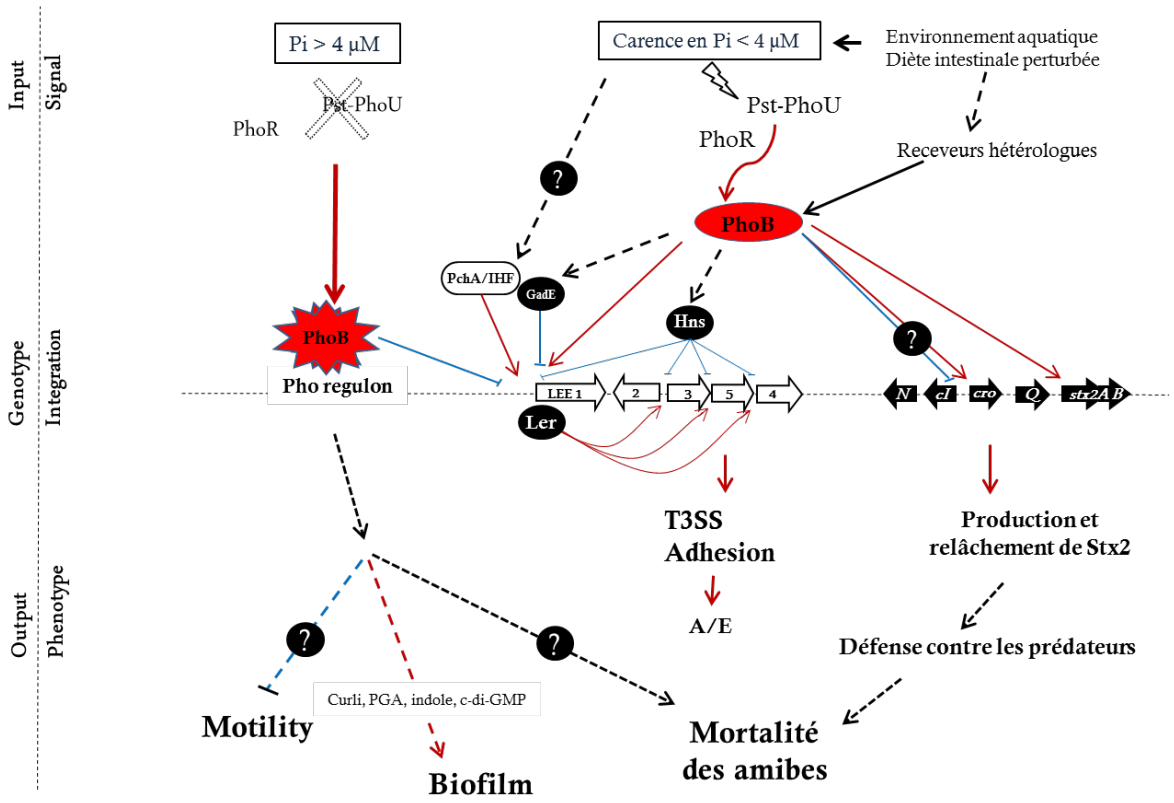


Figure 3. Modèle de régulation de la virulence des EHEC et de ses modes de vie en fonction de la disponibilité du Pi et de l'expression du régulon Pho.

Les lignes solides indiquent les interactions physiques de PhoB avec les régions promotrices et les voies d'induction et de répression avérées. Les lignes discontinues indiquent un effet sans interaction physique ou des voies partiellement élucidées. La couleur rouge indique l'activation tandis que la répression est représentée par la couleur bleu.

- En condition de carence en Pi, PhoB active directement LEE1 qui exprime Ler. Ce dernier active l'expression des opérons LEE2, 3, 4 et 5. Accessoirement et de manière indirecte, dépendante ou pas de PhoB, la carence en Pi augmente l'expression des gènes du LEE à travers l'induction d'autres régulateurs tels que : GadE, PchA, IHF et HNS. Par conséquent, la sécrétion d'effecteurs via le SST3 augmente, conduisant éventuellement au phénotype A/E dans ces conditions. Cependant, en conditions riche en Pi, l'expression constitutive et excessive du régulon Pho (Δ*pst*) entraine une répression générale des gènes du LEE. Ce mutant est également caractérisé par sa capacité à former un important biofilm, par sa mobilité réduite et par son habilité à ralentir la croissance des amibes en co-culture.
- Parallèlement, PhoB lève indirectement la répression du Phage BP-933W par CI permettant l'expression des anti-terminateurs N et Q. Ceci permet l'expression des gènes précoces et retardés qui codent pour les composantes nécessaires à la réplication et l'excision du phage. Les gènes *stx2* sont codés par l'opéron retardé du phage situé en aval du promoteur $P_{R'}$ suggérant leur transcription pendant l'induction du phage. PhoB se lie également à la région promotrice de l'opéron *stx2*. Il n'est pas encore connu si l'activation de *stx2* est due à l'action de PhoB sur leur promoteur ou bien à l'activation de $P_{R'}$. Néanmoins, les Stx permettent aux EHEC de se défendre contre les prédateurs bactériens.

L'analyse de puce à ADN et la RT-PCR quantitative démontrent pour la première fois que le signal de carence en Pi active l'expression des gènes du LEE. Le régulateur PhoB et la carence en Pi, ces deux facteurs sont capables d'augmenter la sécrétion de protéines par T3SS. De plus, PhoB est requis pour l'augmentation de la transcription du LEE2 et LEE3. PhoB donc est un activateur des gènes du LEE et interagit directement avec les promoteurs du LEE1 et LEE2 contenant des boîtes Pho.

La régulation de l'expression du LEE est complexe et implique de nombreux régulateurs positifs et négatifs [557]. Dans la plus part des cas, ces facteurs influencent l'expression du LEE à travers le contrôle direct ou indirect de l'expression du gène *ler*. Cependant, d'autres mécanismes extra-transcriptionnels peuvent interférer avec la régulation de l'expression du LEE [558]. Nos résultats suggèrent que PhoB agit sur l'expression du LEE en se fixant sur le promoteur de *ler*/LEE1, ce qui en retour pourrait activer l'ensemble LEE. En outre, PhoB est impliqué dans l'expression du LEE2 par interaction directe avec son promoteur. Cependant, il est aussi possible que des régulateurs autres que PhoB pourraient être impliqués dans la régulation précise des gènes du LEE. En effet, certains régulateurs positifs du LEE tel que PchA, IHF et GadE sont aussi surexprimés en carence en Pi, tandis que le régulateur négatif HN-S est surexprimé chez le mutant Δ*phoB*.

En revanche, dans un milieu riche en Pi, la surexpression du régulon Pho de manière constitutive (par abolition du système Pst) réprime les gènes du LEE et baisse la sécrétion par le SST3. Chez le mutant Δ*pst*, l'expression du régulon Pho est plus active que chez dans les conditions de carence en Pi. Nous avons montré précédemment que le niveau d'activation du régulon Pho influence le degré d'atténuation de la virulence chez les APEC [455]. Par conséquent, l'expression du LEE est probablement influencée par le niveau d'activation du régulon Pho. En conditions de carence en Pi, le LEE est activé, alors que la mutation Δ*pst*, réprime le LEE.

Des observations similaires ont été rapportées chez *V. cholerae*, où l'expérimentation montre que l'abolition, aussi bien du gène *phoB* que des gènes du système Pst, perturbe la colonisation intestinale chez les souriceaux [417]. Cependant, chez *V. cholerae*, le régulateur PhoB influence indirectement l'expression des gènes de la toxine cholérique (CT), en court-circuitant la cascade de régulation AphA/AphB/TcpPH/ToxB [417].

Par ailleurs, nous avons démontré que plusieurs gènes codés par le bactériophage BP-933W sont aussi modulés en réponse à la carence en Pi. Parmi les gènes activés on retrouve les gènes de régulation du phage, Cro et les anti-terminateurs N et Q, alors que le répresseur CI est réprimé. Habituellement, ce répresseur maintient le prophage en état de quiescence [170]. L'induction du phage est une conséquence de la levée de sa répression phagique lors du cycle lytique. Ultimement, le répresseur CI contrôle la production et/ou libération des toxines Stx2. Chez les EHEC, les résultats de nos expériences montrent que l'expression, la production et le relâchement de la toxine Stx2 augmentent en conditions limitées en Pi. Les facteurs régulant le passage de l'état de quiescence à l'état lytique, tels que répresseurs et sites opérateurs, jouent des rôles importants dans la régulation de la production de Stx [587]. Nos résultats montrent qu'en conditions limitées en Pi, PhoB inhibe indirectement la transcription du gène du répresseur CI, ce qui en retour active l'expression du gène *cro* qui devrait induire le cycle lytique et par conséquent augmenter la transcription du *stx2*. Durant ce processus d'induction du phage, l'anti-terminateur Q se fixe sur le promoteur $P_{R'}$ et en transcendant le terminateur $t_{R'}$ assure la continuité de la transcription des gènes codant Stx2 et ceux codant les fonctions lytiques. Un scénario alternatif serait que PhoB se fixe directement sur les séquences boîtes Pho présentes dans la région promotrice des *stx2* et active leur expression.

L'induction du phage est souvent le résultat d'une réponse SOS suite au dommage de l'ADN bactérien. Cette réponse SOS implique la production d'enzyme telle RecA et LexA [176] (voir détail dans la section 2.5 : régulation des phages Stx2). Dans la présente étude, il semble qu'en réponse aux conditions limitées en Pi, l'induction du phage PB933 est indépendante à RecA et LexA. La carence en Pi et/ou l'effet indirect de PhoB sur l'induction du phage passeraient donc par un ou des facteurs qui restent encore inconnus.

Dans l'intestin, des signaux provenant de l'hôte tels que des changements dans la concentration des métabolites, peuvent permettre aux bactéries de surveiller leur stade infectieux et modifier leur comportement notamment leur virulence. Récemment, Pieper *et al.* [568], ont montré que les EHEC activent le régulon Pho dans l'environnement intestinal de porcelets gnotobiotiques. Le signal conduisant à l'induction du régulon Pho serait vraisemblablement la limitation en Pi. Cependant, nous ne pouvons pas être certains que PhoB peut être régulé par d'autres signaux en plus de la concentration en Pi. En effet, en réponse à d'autres signaux environnementaux, l'activation de PhoB est possible par activation croisée

via des receveurs hétérologues : histidine kinases non-partenaires (Fig.4), telles que CreC, ArcB, KdpD, QseC, BaeS and VanS [419,549,594,595,646].

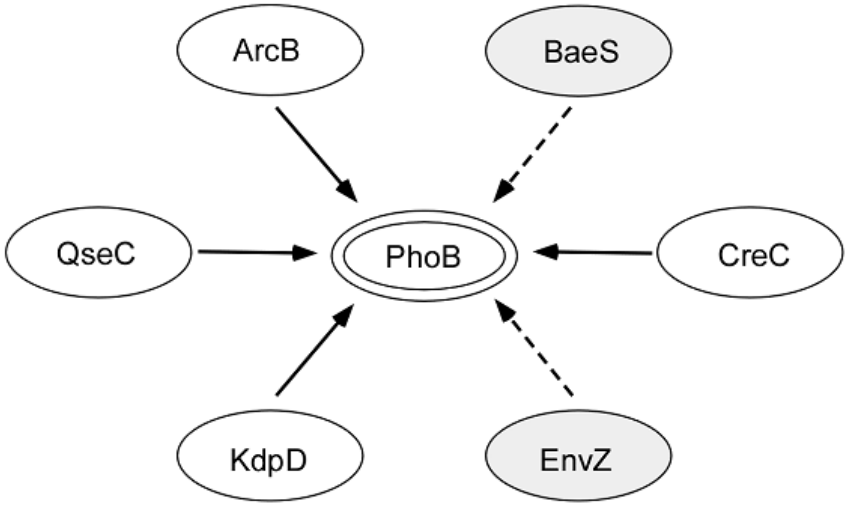


Figure 4. Activation de PhoB par des HKs non-partenaires (Zhou et al. 2005) [646].
Les flèches en continu indiquent les HKs qui activent fortement PhoB. Les flèches en discontinu indiquent les HKs qui activent faiblement PhoB.

c. Implication du régulon Pho dans la virulence d'autres *E. coli* pathogènes

Nous avons déjà recensé les données de littérature concernant les effets de l'induction Pho régulon sur la virulence d'*E. coli* et la relation entre son métabolisme de Pi et sa pathogénicité [338,452]. Dans les 3 dernières années, les études ont continué à décrire les mécanismes phénotypiques et génétiques de virulence d'*E. coli* en fonction de la concentration en Pi environnemental ou de la mutagenèse des systèmes Pho / Pst.

Chez les ExPEC, les études ont montré l'implication du régulon Pho dans la virulence des souches: ExPEC 5131, APEC O78 et UPEC CFT073 causant respectivement la septicémie porcine, la colibacillose aviaire et l'infection du tractus urinaires (UTI) humain. Chez toutes ces souches, l'expression constitutive du régulon Pho par délétion du système Pst attenue leur déterminants de virulence, incluant la sensibilité au sérum et au peroxyde d'hydrogène, la production des fimbriae de type 1 et la modification de la surface cellulaire. Chez la souche

UPEC CFT073, l'expression du régulon Pho altère l'expression des régulateurs *fimB*, *ipuA* et *ipbA* et diminue le niveau cellulaire de l'alarmone ppGpp entrainant une réduction de la production de fimbriae et de colonisation de la vessie murine [457]. Par ailleurs, chez la souche APEC O78, l'activation constitutive du régulon Pho médiée par PhoB est critique pour la virulence, plutôt que l'inactivation du système Pst. En effet, une mutation ponctuelle du gène *phoR*, activant constitutivement le régulon Pho indépendamment du transport de Pi ou du système Pst, a entraîné une atténuation de la virulence *in vivo* [455].

Dans la classe d'*E. coli* pathogènes intestinales, outre les EHEC, il a aussi démontré que le régulon Pho est impliqué dans la modulation de la virulence des EPEC. Ces deux pathovars d'*E. coli* partagent avec *C. rodentium* la capacité de causer un phénotype A/E due au locus LEE codant le SST3 et certains de ces effecteurs. Lors des expériences *in vitro* et *ex vivo*, la délétion du système Pst chez plusieurs souches EPEC, a provoqué leur défaut d'attachement et d'adhésion aux cellules épithéliales. En outre, il a été montré que le mutant pst de *C. rodentium* est moins virulent chez la souris, son hôte naturelle [458], et récemment une étude a démontré que PhoB contrôle directement 2 déterminants de virulence chez *C. rodentium*: la serine protéase DegP et la protéine effectrice du SST3 NleG8 [459].

D. L'expression du régulon Pho favorise un mode de vie en biofilm chez les EHEC

Pendant les conditions défavorables telle que les périodes de famine, les bactéries se regroupent et se développent dans une communauté protégée en s'enfermant dans une matrice de polysaccharides, un processus appelé formation de biofilm. Dans les conditions de laboratoire en culture dans un milieu riche, les bactéries de sérotype O157:H7 forment un faible biofilm [616]. Cependant, en milieu minimal M9 ou MOPS, nous avons montré que la souche EDL933 forme un biofilm qui est 3.6 fois plus important chez le mutant Δ*pst*. Ce mutant «hyper-formateur» de biofilm est aussi hypo motile (Fig. 3). Plusieurs gènes codant pour des composantes impliquées dans la formation de biofilm sont modulés aussi bien chez ce mutant Δ*pst* qu'en réponse à une carence en Pi chez la souche sauvage. Parmi ces gènes on retrouve principalement les gènes de biosynthèse de curli, PGA, production d'indole et de c-di-GMP.

La relation entre la disponibilité du Pi et la formation de biofilm à été investiguée chez d'autres espèces bactériennes, notamment *A. tumefaciens*, un pathogène de plantes qui forme plus de biofilm en milieu pauvre en Pi en augmentant son attachement par le polysaccharide unipolaire (UPP) contrôlé par le système PhoB/R [462,463]. En revanche, chez *Proteus mirabilis* qui cause des UTIs, l'abolition du système Pst entraîne un déficit de formation de biofilm dans un milieu à base d'urine humaine [467]. Chez *V. cholerae* cultivée en milieu pauvre en Pi, une corrélation positive entre le niveau cellulaire du c-di-GMP et le taux de formation de biofilm à été observée. Ce changement phénotypique est dû au contrôle direct par PhoB du gène *acgA*, codant pour une phosphodiesterase contenant un domaine EAL et dégradant le c-di-GMP [433]. De manière similaire, chez *P. aeruginosa* la limitation en Pi entraîne une augmentation de production de rhamnolipids, qui sont des biosurfactants qui induisent la motilité et réduisent la formation de biofilm [448]. En outre chez *P. fluorescens*, la formation de biofilm requiert la production de l'adhésine LapA. Cette adhésine est stimulée par le niveau cellulaire de c-di-GMP. Cependant, lorsque *P. fluorescens* est en carence en Pi, elle perd sa capacité à former un biofilm à cause de l'induction par PhoB de l'activité PDE de l'enzyme LapD, entraînant une baisse intracellulaire de c-di-GMP [450].

Il semblerait donc, que le rôle de la régulation homéostatique en Pi dans le phénotype de formation de biofilm bactérien est multifactoriel et variable suivant les méthodes et souches bactériennes investiguées. Néanmoins, nos résultats soulignent l'importance des facteurs extracellulaire (Pi) et intracellulaire (induction de Pho) dans la formation de biofilm chez les EHEC, ce qui pourrait avoir un impact sur sa transmission et sa virulence.

Conclusions et perspectives

Les études conduites dans cette thèse nous permettent de décrire un mode de vie possible d'EHEC qui peut contribuer à la compréhension de son écologie et conduirait à des stratégies possibles pour lutter plus efficacement contre sa transmission aux humains et sa réintroduction chez les ruminants. Nous avons démontré le potentiel de l'amibe *A. castellanii* à héberger les EHEC et à contribuer à leur persistance dans l'environnement.

En plus d'être un réservoir potentiel pour les EHEC et par ce qu'elle présente des similitudes avec les macrophages, cette amibe pourrait aussi jouer le rôle d'entraineur des EHEC pour faire face aux cellules immunitaires lorsqu'elles sont transmises à l'humain. Compte tenu du petit nombre d'EHEC suffisant pour l'infection humaine, le rôle des protozoaires dans la contamination de l'eau et de la nourriture exige davantage de recherche en matière de prévention.

En perspective, il serait donc judicieux d'évaluer cette association EHEC/amibes dans le réseau d'eau potable et dans les effluents d'abattoirs ou de fermes où il y a le plus de concentrations des EHEC. Cela pourrait déboucher sur des moyens de prévention des contaminations de l'eau par l'industrie agricole. Concernant la toxicité des EHEC sur les amibes, Il serait pertinent de mesurer et d'investiguer l'éventuel mécanisme d'entrée de Stx dans la cellule amibienne ou de son relâchement intracellulaire. Un niveau basal de toxicité sur les amibes, indépendant des Stx, a été constaté en présence de *E. coli* non-pathogène. Cela pourrait dû aux LPS ou à autres composés.

Également en perspective, en utilisant la technique d'immunoprécipitation de la chromatine, il serait intéressant d'examiner *in vivo* l'effet de la carence en Pi via PhoB sur l'expression des gènes du LEE et du phage BP933W. L'expression de ces deux facteurs de virulence est-elle modulée différemment en fonction du niveau d'expression de PhoB et/ou de la concentration du Pi ? Pour répondre à cette question, il faudrait tester comparativement des souches EHEC dont l'opéron *phoBR* est sous le contrôle de promoteur inductible (ex : à l'IPTG), constitutif et natif. En variant la concentration en Pi et en même temps en contrôlant le niveau d'induction du régulon Pho, nous serons capables de déterminer les conditions d'activation de ces facteurs de virulence. Également, pour comprendre si l'effet de PhoB sur les gènes du Phage BP933W, se traduit seulement par une hausse d'expression et de production des toxines Stx2 ou s'accompagne par une production de particules phagiques et

lyse cellulaire, nous envisagons d'ultracentrifuger les surnageants de nos cultures cellulaires après les avoir traiter à la DNase et RNase et ensuite réaliser des PCR quantitative sur les gènes *stx* et d'autres de morphogenèse.

Les résultats de ce projet sont également compatibles avec le potentiel du TCS contrôlant le régulon Pho comme une cible potentielle et alternative aux antibiotiques pour lutter contre l'infection par les EHEC. En effet, en plus de la problématique concernant la résistance bactérienne aux antibiotiques, le cas des EHEC est plus soucieux puisque certains antibiotiques provoquent la réponse SOS des phages portant les gènes *stx*. En ce sens il serait intéressant de développer des approches novatrices telles que l'usage de modulateurs spécifiques du TCS PhoB/R. Cependant, ces stratégies nécessitent plus de prudence du fait que d'après nos résultats, l'expression des gènes virulence chez les EHEC peut être opposée suivant le niveau d'expression du régulon Pho. Une autre stratégie donc serait de tester des souches EHEC Pho constitutives comme candidats potentiels pour concevoir des vaccins atténués puisque le mutant *pst* montre un niveau d'expression du LEE très bas. Par conséquence, le niveau d'atténuation de virulence peut être contrôlé par le type de mutations au niveau des opérons *phoBR* et *pstSCAB-phoU*.

En outre, les analyses transcriptomiques et la délétion du système Pst entraînant un phénotype hyper-biofilm suggèrent que les EHEC présentent dans des environnements limités en Pi, en formant plus de biofilm, peuvent donc constituées un risque significatif de contamination continue. Dans un premier temps, il serait judicieux de savoir si cela implique le régulateur PhoB en testant le double mutant Δ*pst*Δ*phoB*. S'il s'avérait que la formation du biofilm est PhoB-dépendante chez les EHEC, la suite serait de comprendre comment en recherchant les gènes reliés aux biofilms et qui seraient directement ou indirectement régulés par PhoB.

Ainsi, pour la gestion du risque de sa transmission, il serait intéressant de surveiller le niveau de Pi dans l'eau et additifs alimentaires et les futures études pourront déterminer le seuil de concentration en Pi, en dessous duquel les EHEC forment un biofilm dans les aliments et dans leurs étapes de préparation. Également en perspective, il faudrait évaluer la survie des EHEC à une carence en Pi dans les milieux aquatiques dans lesquels nous pouvons les détecter, et vérifier l'effet de ces conditions sur ses facteurs de virulence.

Les bactéries *E. coli* pathogènes et commensales sont équipées pour faire face et s'adapter aux limitations nutritionnelles. Le signal de carence en Pi est directement intégré par les systèmes Pst et PhoB-R permettant une réponse d'homéostasie de Pi et un réajustement métabolique et énergétique. Cependant, les pathogènes tels que les EHEC utilisent ce même signal pour moduler leur traits de virulence et leur mode de vie. En effet, suivant le niveau du Pi disponible et l'expression du régulon Pho, les EHEC modulent l'expression génique des voies impliquant les Stx, les adhésines et effecteurs sécrétés par le SST3 et probablement d'autres composantes. Cela se traduirait par une variation de leur virulence et des changements adaptatifs comme la cytotoxicité, la colonisation intestinale et la formation de biofilm.

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Annexe 1

Article de revue # 3 «The Pho regulon and the pathogenesis of *Escherichia coli»*

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Abstract

During the course of infection, bacteria must coordinately regulate gene expression in response to environmental stimuli. The phosphate (Pho) regulon is controlled by the two component-regulatory system PhoBR. PhoBR is activated during starvation and regulates genes involved in phosphate homeostasis. Several studies have highlighted the importance of the Pho regulon in bacterial pathogenesis, showing how induction of PhoBR, in addition to regulating genes participating in phosphate metabolism, leads to modulation of many cellular processes. The pleiotropic effects of Pho regulon activation include attenuated virulence and alteration of many virulence traits, including adhesion to host cells and resistance to cationic antimicrobial peptides, acidity and oxidative stresses. This review provides an overview of the relationship between the Pho regulon and virulence in Escherichia coli and illustrates that, in addition to regulating phosphate homeostasis, the Pho regulon plays a key role in regulating stress responses and virulence.

Keywords

Pho regulon; Pathogenic Escherichia coli; Two-component regulatory system; Stress response

1. Introduction

To adapt and survive in different microenvironments, bacteria must sense and respond to extracellular signals. The adaptive response to environmental stimuli can be transduced by two-component regulatory systems [647], which are involved in the regulation of chemotaxis, osmoregulation, metabolism and transport [648]. A typical two-component regulatory system (TCRS) is composed of an inner-membrane histidine kinase (HK) sensor protein and a response regulator (RR) that acts as a DNA-binding protein, activating or repressing gene expression [649].

Phosphorus, in terms of cellular content, is the third most abundant element. It is found in several molecules, including membrane lipids, complex sugars and nucleic acids. Phosphate is also involved in energy metabolism and in signal transduction, which is mediated by a TCRS [650]. The extracellular concentration of phosphate is sensed by the two-component regulatory system PhoBR, in which PhoR encodes the HK and PhoB the RR. PhoBR responds to phosphate limitation, when the extracellular phosphate concentration falls below 4 μM. In phosphate-limiting conditions, PhoBR induces genes belonging to the Pho (phosphate) regulon, which includes genes involved in acquisition and metabolism of different phosphate groups [338,404,650]. The control of the Pho regulon and transmembrane signal transduction by environmental inorganic phosphate (P_i) has been extensively studied in *Escherichia coli* and *Bacillus subtilis.* In *E. coli* K-12, the Pho regulon comprises 31 genes [404]and, in addition to being involved in phosphate homeostasis, is also connected to bacterial virulence as its induction results in attenuated pathogens.

Host–pathogen interactions are dynamic processes responding to the diverse environmental conditions encountered by invading pathogens. Survival of a pathogen in different sites in the host requires an adaptive response capable of reacting to different stimuli in its immediate environment. The specialised regulatory systems that control the expression of virulence factors are essential for survival and necessarily complex, with interconnections between regulatory systems at many levels. Although the presence of pathogen-specific genes may dictate the pathogenic lifestyle and virulence potential of pathogenic *E. coli*, products encoded by conserved or “core” genes undoubtedly contribute to functional metabolism, physiology

and adaptation to environmental changes, including host environments and resistance to host defences.

Lamarche et al. [338]reviewed the relationship between the Pho regulon, metabolism and pathogenicity. Here, we present a review specifically focusing on the effects on virulence of *Escherichia coli* by the induction of the Pho regulon. First, we briefly overview the induction of the Pho regulon, then discuss the different virulence attributes affected by the induction of the Pho regulon of pathogenic *E. coli*, including resistance to oxidative stress, membrane perturbation, production of adhesins and adaptation to environmental stimuli.

2. Induction of the Pho regulon

As mentioned above, phosphate starvation is sensed by the TCRS PhoBR. PhoBR is activated when the extracellular phosphate concentration falls below 4 μM, inducing transcription of genes belonging to the Pho regulon. This is enabled by the binding of phosphorylated PhoB to specific DNA sequences, known as Pho boxes, located within Pho-dependent promoter regions. PhoB binding either induces or represses transcription of genes that comprise the Pho regulon, including those involved in phosphate transport and metabolism [404,650] (Fig. 1). One of these systems, the*pstSCAB–phoU* operon, encodes the phosphate-specific transport (Pst) system. The Pst system encodes an ATP-binding cassette (ABC) transporter involved in the transport of inorganic phosphate (P_i). In addition to acting as a phosphate transporter, the Pst system is required for P_i signal transduction, as mutations in any of the genes of the *pst* operon result in constitutive expression of the Pho regulon, regardless of environmental phosphate availability [338,404,650] (Fig. 1).

In addition to regulating genes involved in phosphate acquisition and metabolism, induction of the Pho regulon seems to have pleiotropic effects, as proteomic analysis of *E. coli* K-12 cultured under phosphate-limiting conditions reveals that up to 400 proteins are differentially expressed [339]. Microarray experiments performed on K-12 and pathogenic *E. coli* connected the Pho regulon directly or indirectly with multiple metabolic systems [338,550,636,651] (Table I). Notably, the Pho regulon and bacterial virulence are connected, as its induction regulates pathogen-specific genes, and affects survival and virulence of many bacterial pathogens [338,417,455,456,458,508,652-654]. Multiple stress responses and virulence attributes are affected by inactivation of the Pst system. Specifically, general, oxidative and

acid stress responses are connected with the Pho regulon. The connection between the general and acid stress responses has consequences for the virulence of pathogenic *E. coli* [338,456]. Among the virulence attributes altered by induction of the Pho regulon are a significant reduction in the amount of capsular antigen at the cell surface, resistance to the bactericidal effect of serum, to cationic antimicrobial peptides, and to acid and oxidative stresses, as well as the production of type 1 fimbriae [453-456,508,569,655].

3. Pho regulon activation and ExPEC virulence

Extra-intestinal pathogenic *E. coli* (ExPEC) are an important group of pathogenic *E. coli* that cause a diversity of infections in both humans and animals, including urinary tract infections (UTIs), meningitis, and septicaemia [656,657].

The role of the Pho regulon in virulence of ExPEC has been principally studied in the ExPEC strain 5131, which causes septicaemia in pigs, and the avian pathogenic *E. coli* (APEC) O78 strain χ7122, which causes colibacillosis in poultry [453-456,508,569]. In APEC strain χ7122, the level of attenuation of virulence of mutant strains correlated directly with the level of activation of the Pho regulon [455]. However, selective capture of unique transcribed sequences (SCOTS) revealed that *phoB* was expressed during experimental infection of chicken with APEC strain χ7122 [484]dozois, suggesting that fine-tuning of the Pho regulon is required for virulence, while inappropriate or constitutive induction of the Pho regulon has deleterious effects.

4. Dissecting the specific contribution of both PhoBR and Pst systems

As mentioned above, the Pst system contributes to both regulation of the Pho regulon and high-affinity uptake of P_i. Until recently, this dual function for Pst made it difficult to explain the effects of Pst inactivation on the virulence of APEC, as it was unclear whether attenuation was due to constitutive activation of the PhoBR TCRS or the loss of Pst-mediated high-affinity phosphate uptake. However it has been shown that PhoB-mediated constitutive activation of the Pho regulon, rather than inactivation of the Pst system, was critical for the virulence of APEC [455]. A point mutation in *phoR*, which constitutively activated the Pho regulon independently of P_i transport and inactivated the *pst* system, attenuated virulence and virulence attributes, such as sensitivity to hydrogen peroxide and serum, and production of type 1 fimbriae [455]. Interestingly, it was determined that the PhoB regulator is not required

for virulence in APEC. This is contrast with the implications of SCOTS analysis experiments, but accords with recent work by Pratt et al. [417] that showed that the attenuation of virulence of *V. cholerae*in a *pst* mutant was due to induction of the Pho regulon, not P_i transport per se.

5. Oxidative stress response

Oxidative stress is produced by bacterial metabolism (respiration), the immune system and exposure to host environmental factors such as metal ions. Oxidative stress induces damage to DNA, proteins and membranes, and can lead to cell death [658]. The expression of some genes whose products exhibit antioxidant activities were modulated in an APEC *pst* mutant [456]. The catalase*katE*, the superoxide dismutase *sodC*, the DNA protection protein *dps* and the small regulatory RNA *oxyS*were among the differentially expressed genes. However, the *pst* mutants were more sensitive to agents generating reactive oxygen intermediates (ROI) than the parent strain χ7122 [455,456]. In *E. coli* K-12, it was observed that the alkylhydroperoxide reductase*ahpCF*, the catalase *katG* and the pyruvate oxidase *poxB* genes are required to resist oxidative stress generated by glucose metabolism [659,660]. Since the ability to resist oxidative stress is crucial for full virulence in ExPEC [661], the sensitivity of *pst* mutants to oxidative stress could explain, at least in part, their attenuation.

6. Bacterial cell surface modification

Under conditions of phosphate starvation, bacteria have been shown to modify their phospholipids by substituting them with phosphorus-free lipids [338,662]. A strong influence of the Pst system and the associated Pho regulon in modifications of lipid A structure and cell surface perturbations has been demonstrated in strain χ7122. Indeed, mutation in the Pst system results in structural modifications of lipid A, including a reduced amount of its hexa-acylated-1-pyrophosphate form [569]. The outer monolayer of the outer membrane of most gram-negative bacteria is composed of lipopolysaccharides, which includes the lipid A. These are involved in outer membrane integrity, forming a protective barrier against various environmental stresses [663-666]. The change in lipid A structure in *pst* mutants is exemplified by increased sensitivity of the mutant to serum complement, vancomycin and cationic antimicrobial peptides (CAMPs) [454,455,508,569].

In addition to being involved in the biosynthesis of lipid A, deletion of the Pst system leads to an imbalance in cyclopropane (CFA) and unsaturated fatty acids (UFA), and increases outer

membrane permeability [453]. Microarray analysis has also revealed that lipid A modification and the differential expression of genes belonging to the biosynthesis of enterobacterial common antigen and LPS biosynthesis occurred in a *pst* APEC mutant [456,569]. Since membrane integrity is required for resistance to environmental stresses, membrane perturbation in Pst mutants may explain the decrease of such virulence traits in pathogenic *E. coli*.

7. Adhesin production and adherence

Type 1 and F9 fimbriae mediate adherence of *E. coli* to host cells and are involved in biofilm formation [667-669]. Type 1 fimbriae play a key role in virulence [670-675]. In APEC *pst* mutants, it was observed that expression of these fimbriae was repressed [455,456], as no fimbriae were found on their surface [456]. Since type 1 fimbriae were preferentially expressed in air sacs, the primary site of infection of APEC [667,676], a decrease in fimbrial production in the *pst* mutant may contribute to reduced APEC colonisation and virulence. Similarly, in uropathogenic *E. coli* (UPEC), inactivation of the *pst* system also repressed expression of type 1 fimbriae and altered virulence (Crépin et al., manuscript in preparation).

8. Intestinal pathogenic *E. coli*

Intestinal pathologies mostly result in more or less severe diarrhoea, in some cases leading to more severe diseases. Among the diseases associated with *E. coli* include enteric infections caused by different pathotypes of diarrhoeagenic *E. coli*, such as enterotoxigenic, enteropathogenic and enterohaemorrhagic*E. coli* (ETEC, EPEC and EHEC respectively). Diarrhoeagenic EPEC and EHEC produce a characteristic attaching and effacing (A/E) lesion on the brush border of infected intestinal enterocytes that is characterised by localised destruction (effacement) and intimate bacterial attachment [9]. These pathotypes pose a significant risk to human and animal health world-wide. As a cause of animal (and zoonotic) disease, attaching and effacing *E. coli* (AEEC) infections have a great impact on human food safety (with animals such as cattle reservoirs of some human pathogenic strains), animal welfare, economic production (costly outbreaks of post-weaning diarrhea in pigs, risk of trade barriers), and environmental biosafety.

9. Pst system and adhesion of intestinal pathogenic *E. coli* strains

In the *in vitro* organ culture (IVOC) model, insertion of a transposon into the *pstS* gene impairs the ability of a porcine EPEC strain to attach to piglet ileal enterocytes [677]. Deletion of the *pst* operon in the EPEC strain LRT9 (O111:abH2) impairs its adherence to Hep-2 intestinal cells [652]. It has been hypothesised that the reduced adherence was due to the down-regulation of the *bfp*operon and *eae*, which code for the bundle forming pili and the adhesin intimin, respectively. Bfp is involved in the formation of microcolonies and intimin is involved in intimate adherence [652]. Their positive regulator genes, *perA* and *perC*, were also repressed [652]. Similarly, in the atypical EPEC strain E128012 (O114:H2), which lacks Bfp, adherence of a *pst* mutant to Hep-2 and T84 intestinal cells was reduced [458]. A pst mutant of the pathogen *Citrobacter rodentium*, which causes attaching and effacing intestinal lesions in mice, is excreted in lower numbers by C56BL/6 mice [458]. It was suggested that the Pho regulon represses uncharacterized adhesins required for virulence [458]. Indeed, activation of the Pho regulon (PhoBR) by deletion of the*pst* system represses these adhesins, while adherence to HT-29 intestinal epithelial by EHEC O157:H7 ATCC strain 43894 downregulates *phoBR* and *pstS* [678].

These observations indicate that disruption of the *pst-phoU* locus not only activates the Pho regulon but also induces various effects on adherence. In EPEC and EHEC, an intact Pst system seems to be required for full virulence by facilitating intestinal colonization.

10. Activation of the Pho regulon during adaptation by EHEC to environment

The role of the Pst system (Pho regulon) in the virulence of EHEC has not been well studied. Information about the induction of the Pho regulon by environmental stress mainly emanates from transcriptional studies. Exposure of *E. coli* O157:H7 strain EDL933 to sodium benzoate, a bacteriostatic and fungistatic agent, led to the up-regulation of *pst* and other genes of the Pho regulon [679]. It was suggested that the Pst system could function as an efflux pump for sodium benzoate, as observed for*Mycobacterium smegmatis* [680]. Exposure of *E. coli* O157:H7 strains TW14359 and Sakai to oxidative stress up-regulated transcription of the *pst* operon as well as PhoBR [681]. Under acid shock conditions, the Pho regulon response regulator *phoB* was induced in EHEC O157:H7 strain FDA518 [682]. PhoB activates *asr*, a

gene encoding an acid shock protein required for survival in moderately acidic conditions in *E. coli* K-12 [594], and to induce other acid shock proteins [456,550,651]. Using *in vivo*-induced antigen technology (IVIAT), PhoE and PhoA, an outer membrane porin involved in the transport of various anions and a periplasmic alkaline phosphatase, respectively, were identified among the *E. coli* O157:H7 proteins reacting with sera from patients previously diagnosed with haemolytic and uraemic syndrome [683]. This further indicates that members of the Pho regulon are expressed by pathogenic *E. coli* during infection.

11. EHEC virulence factors regulated by PhoBR

Candidate genes with putative PhoB binding sites were identified in EHEC O157:H7 Sakai as well as strain EDL933 [654]. In the Sakai strain, one of these loci was confirmed to be directly regulated by PhoB using a genomic library fused to *lacZ*. Yoshida et al. [593] have demonstrated that the gene cluster*esc0540-0544* is positively regulated by the Pho regulon. This gene cluster is homologous to the *siiCA-DA*operon of *Escherichia fergusonii*, which encodes a putative RTX toxin and its cognate type I secretion system (T1SS) [593]. Although the function of this putative RTX toxin in *E. coli* O157:H7 has not yet been established, it was suggested that P_i sensing could regulate such a secretion system in EHEC.

12. Concluding remarks

Pathogenic *E. coli* must survive in nutritionally disparate environments, including some ecosystems where P_i can be limited [417]. The Pho regulon is required for survival, not only to control phosphate homeostasis, but also to take part in a complex network important for both bacterial virulence and stress responses. Inappropriate expression or repression of this system may have significant consequences on bacterial virulence. Mutations in the Pst system or PhoB result in multiple effects, including alteration of *E. coli* virulence traits. Although these effects may be due to a deficiency in phosphate uptake under some conditions, PhoBR TCRS regulation is likely to be responsible for the multiple effects that are observed in Pst and PhoB *E. coli* mutants. PhoB-mediated constitutive activity of the Pho regulon plays a major role in attenuation of virulence and associated traits in these mutants. Changes in adhesin expression, as well as altered capacity to adhere to cells and tissues, are affected by the activation of the Pho regulon. More in depth investigation needs to be done to characterize the

functions associated with virulence genes of *E. coli* that are under the control of the Pho regulon.

Every year, the poultry industry sustains significant financial losses due to the high morbidity and mortality caused by APEC. Some ExPEC strains are capable of infecting both poultry and mammals, suggesting the zoonotic potential of certain APEC strains [657,684,685]. EPEC and EHEC infections have an impact on human food safety, animal welfare, economic production and environmental biosafety. With antimicrobial resistance of bacterial pathogens on the increase, it is important to develop novel preventive and therapeutic strategies. Designers of new attenuated vaccine strains may be able to take advantage of the fact that the degree of attenuation in *pst* and *phoBR* mutants can vary with the degree of constitutive activation of the Pho regulon [455]. Drugs inducing the Pst system or directly affecting PhoBR activity may also be useful as therapeutic agents to compromise bacterial virulence and facilitate elimination of the pathogen by host defences [686-691].

Conflict of interest statement

The authors declare no conflict of interest.

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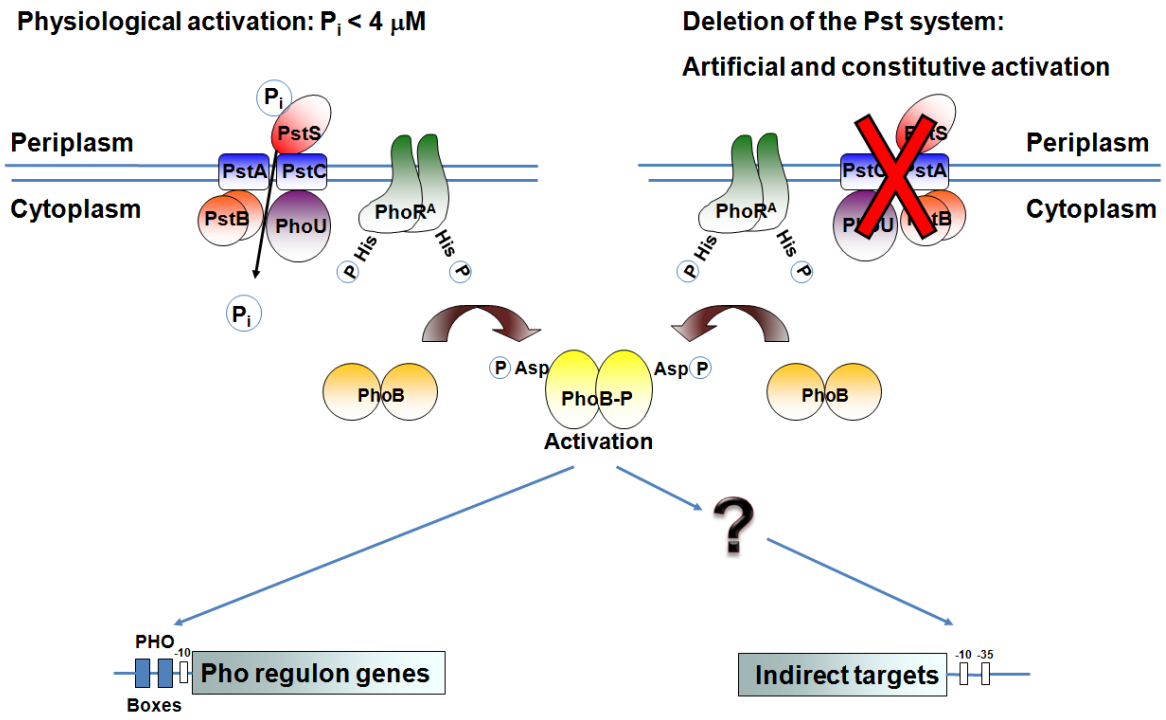

Figure 1. Induction of the Pho regulon by phosphate starvation and inactivation of the Pst system

Adapted from Lamarche *et al*. [569], with kind permission of John Wiley and Sons. Under phosphate starvation, PhoR autophosphorylates on histidine residue. The phosphoryl group is then transferred onto aspartate residue of PhoB. This phosphotransfer activates PhoB which can bind to Pho boxes and activates transcription of Pho regulon genes. PhoB can also act indirectly on gene expression by regulating unknown regulators. Furthermore, mutations in any of the genes of the *pst* operon result in constitutive expression of the Pho regulon, regardless of environmental phosphate availability. Abbreviations: P_i, inorganic phosphate; PstS, periplasmic P_i-binding protein; PstA et PstC, integral membrane channel proteins; PstB, ATP-binding protein; PhoU, regulatory protein; PhoB, RR; PhoR, sensor protein (A, autophosphorylated; P, phosphorylated).

Table I. Attributes affected by the induction of the Pho regulon

Strains	Virulence	Condition[a]	Systems affected	Phenotype	References
K-12					
MG1655	NA		Oxidative stress	Sensitive	[659,660]
	NA		Acid stress	Sensitive	[659]
W3110	NA		Acid stress	NA	[550,651]
ANCK10	NA		Acid stress	NA	[594]
ExPEC					
ExPEC 5131	Attenuated		Cell surface modification	Serum and CAMPsb sensitive	[453,454,569]
APEC χ 7122	Attenuated		Oxidative stress	Sensitive	[455,456]
			Acid stress	Sensitive	[485]
			Type 1 and F9 fimbriae	Afimbriate	[455,456]
			Cell surface modification	Serum and CAMPs[a] sensitive	[455,456]
CFT073	Attenuated		Type 1 fimbriae production	Reduce mouse bladder colonization	Crépin *et al*. manuscript in preparation

Strains	Virulence	Condition[a]	Systems affected	Phenotype	References
EPEC					
LRT9	NA		Adhesin production	Impaired adhesion onto HEp-2 cells	[652]
86-1390	NA		?	Impaired attachment onto piglet ileal explants	[677]
E128012	NA		Adhesin production	Impaired attachment onto HEp-2 cells	[458]
C. rodentium ICC169	Attenuated			Impaired colonization of intestine	[458]
EHEC					
EDL933	NA	Exposition to sodium benzoate	Pst system; PhoBR		[679]
TW14359	NA	Exposition to oxidative stress	Pst system		[515]
Sakai	NA	Exposition to oxidative stress	Pst system; PhoBR; PhoA		[515]
			Putative RTX-toxin protein; Cognate type I secretion system		[593]
FDA518	NA	Acid stress	PhoB		[221]
Isolated from HUS[c] patient	NA	Exposition to convalescent sera	PhoE; PhoA		[195]

[a]Condition in which the Pho regulon is induced; [b] CAMPs, cationic antimicrobial peptides; [c]***Hemolytic-uremic syndrome***