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Interactions bactéries-hôte dans l'axe intestin-cerveau

Nathalie Connil

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UNIVERSITE DE ROUEN
Ecole doctorale Normande, Biologie Intégrative, Santé et Environnement

RAPPORT D'ACTIVITE SCIENTIFIQUE

Présenté par

Nathalie Connil

Maitre de conférences

Spécialités Microbiologie, Biologie cellulaire

Et soutenu le 27 Novembre 2015

Pour l'obtention

DE L'HABILITATION A DIRIGER DES RECHERCHES

Interactions bactéries-hôte dans l'axe intestin-cerveau

Membres du jury

M. Djamel Drider, Professeur, Université de Lille

Mme Pascale Serror, Directrice de recherche, INRA de Jouy en Josas

Mme Sophie Sablé, Maître de conférences-HDR, Université de La Rochelle

M. Joël Fleurence, Professeur, Université de Nantes

Mme Sylvie Chevalier-Laurency, Professeur, Université de Rouen

M. Marc Feuilloley, Professeur, Université de Rouen

Rapporteur

Rapporteur

Rapporteur

Examineur

Examineur

Examineur

-Remerciements-

Je vais commencer par remercier tout d'abord les personnes qui ont bien voulu accepter la lourde tâche d'évaluer ce mémoire et de participer à mon jury de HDR.

Un grand merci à mes rapporteurs, Djamel Drider, professeur à l'université de Lille, Sophie Sablé, maître de conférences-HDR à l'université de La Rochelle, et Pascale Serror, directrice de recherche à l'INRA de Jouy en Josas. J'espère que nous aurons bientôt l'occasion de démarrer ou poursuivre de fructueuses collaborations.

Merci à Joel Fleurence, professeur à l'université de Nantes, d'avoir accepté de participer en tant qu'examinateur dans mon jury de HDR, et de pouvoir découvrir ainsi « mon univers scientifique » et mes amies les bactéries....

Merci à mes examinateurs internes à l'université de Rouen, Sylvie Chevalier-Laurency et Marc Feuilloley, d'avoir réussi à trouver des créneaux communs dans vos emplois du temps respectifs pour qu'on puisse organiser ce jury. Ce n'était pas gagné !! Mais on a réussi !!!

Une énorme pensée pour mes doctorants (Amar Madi, Charlène Leneveu et Kelly Biaggini) et étudiants de master 2 (Ziad Alnabhani, Valérie Borrel), qui ont dû supporter mes exigences, et sans qui ce travail n'aurait pas pu être effectué. Je n'oublie pas non plus tous les stagiaires de courte durée qui ont apporté chacun à leur manière une petite pierre à cet édifice.

Merci à Sylvie Chevalier-Laurency et Emeline Bouffartigues-Maillot pour les petits cafés-dosette au bâtiment C, mais la prochaine fois... je voudrais qu'on invite aussi Georges Clooney !! Sylvie, j'apprécie ton enthousiasme et ton écoute. Toujours présente pour « motiver les troupes » !!!

Je remercie également tous les autres membres du laboratoire. On ne peut pas citer tout le monde... on est de plus en plus nombreux !! La « famille » ne cesse de s'agrandir !!! Merci aux directeurs successifs du laboratoire pour cette belle évolution... Et bientôt des locaux tous neufs !! J'en profite pour remercier mes collègues, Olivier Lesouhaitier et Marc Feuilloley de m'avoir aussi bien accueilli au moment de ma prise de fonction et d'avoir trouvé une petite place pour moi dans leur bureau. Vous allez me manquer quand on va déménager !!

Je dois énormément à Marc Feuilloley de m'avoir sélectionnée sur le poste de maître de conférences, il y a déjà bientôt 10 ans.... pour développer l'axe bactéries-intestin au sein du laboratoire. Oui les recrutements externes parfois ça existe !! Merci de cette jolie opportunité !!

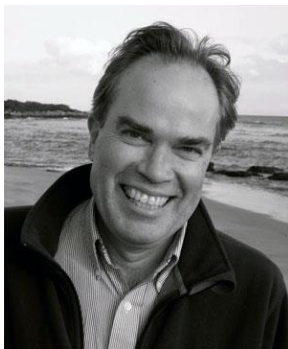
Un grand merci à ma famille et mes amis (ils se reconnaîtront...) pour lesquels je ne suis hélas jamais très disponible.... la recherche ça prend du temps... les cours aussi... Oui maman je rédige encore un « gros livre ».... Un peu comme celui de ma thèse... non papa ce n'est pas pour devenir directeur de laboratoire !! Aha ha

Enfin merci à ceux qui partagent mes cours et stages de danse (zumba, street jam, samba, danse orientale, danse classique..) depuis maintenant 3 ans...

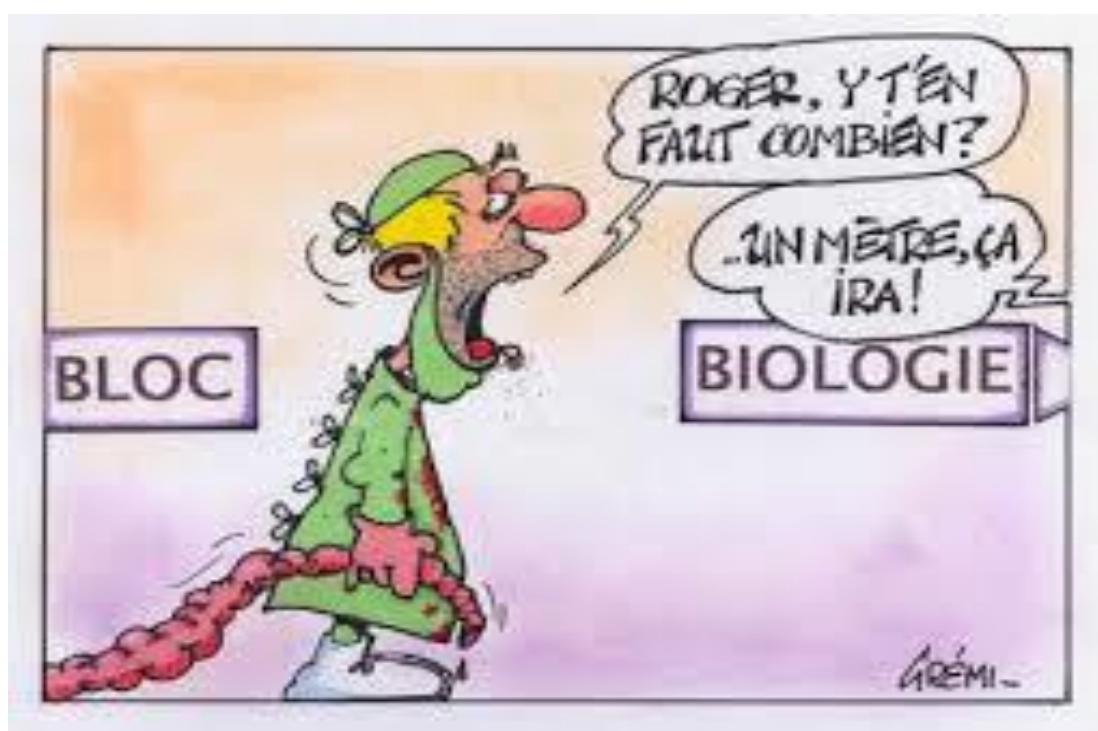
Rien de mieux pour décompresser !!!

Microbes: The Trillions of Creatures Governing Your Health

« We tend to think that we are exclusively a product of our own cells, upwards of ten trillion of them. But the microbes we harbor add another 100 trillion cells into the mix. The creature we admire in the mirror every morning is thus about 10 percent human by cell count. By weight, the picture looks prettier (for once): Altogether an average adult's commensal microbes weigh about three pounds, roughly as much as the human brain. And while our 21,000 or so human genes help make us who we are, our resident microbes possess another eight million or so genes, many of which collaborate behind the scenes handling food, tinkering with the immune system, turning human genes on and off, and otherwise helping us function. John Donne said "no man is an island," and Jefferson Airplane said "He's a peninsula," but it now looks like he's actually a metropolis. »



By Richard Conniff
In Smithsonian Magazine, May 2013



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1) CURRICULUM VITAE

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Née le 16 Avril 1973 (42 ans)

Domicile : 17, rue du moulin vieux- 27000 Evreux

Tél : 06-13-82-53-50

Adresse professionnelle :

Laboratoire de Microbiologie Signaux et Microenvironnement (LMSM), EA4312,
55, rue st Germain 270000 Evreux

E-mail : nathalie.connil@univ-rouen.fr

Situation professionnelle à l'université de Rouen

Octobre 2012 : Obtention de la Prime d'Excellence Scientifique (PES)

Septembre 2007 : Titularisation sur le poste de Maître de Conférences

Septembre 2006 : Recrutement sur le poste de Maître de Conférences, Section CNU 65,
n°emploi 1447 Biologie cellulaire, Microbiologie Cellulaire.

Laboratoire de Microbiologie du Froid, Université de Rouen/IUT d'Evreux

Diplômes-Prix

2003 : Prix Jeune Chercheur (Institut Français pour la Nutrition)

2001 : Doctorat de Microbiologie (Nantes)

Travaux de recherche effectués au Laboratoire de Microbiologie Alimentaire et Industrielle (LMAI, ENITIAA) sous la direction des Prs Hervé Prévost et Xavier Dousset

Thèse soutenue le 10 décembre 2001, mention très honorable et félicitations du jury

Titre : Etude physiologique et caractérisation génétique préliminaire de la production de tyramine par *Carnobacterium divergens* V41, souche au potentiel bioprotecteur dans le saumon fumé.

Président du jury : M. Jean-Noël Hallet (Professeur, Université de Nantes)

Rapporteurs : M. Jean-Baptiste Millière (Professeur à l'ENSAIA, Nancy)
Mme Marie-Christine Montel (Directeur de Recherche INRA, Aurillac)

Examineurs : M. Alain Rincé (Maître de Conférences, Université de Caen)
M. Xavier Dousset (Professeur à l'ENITIAA, Nantes)
M. Hervé Prévost (Professeur à l'ENITIAA, Nantes)

1997 : DEA de Microbiologie Fondamentale et Appliquée (Rennes)

Stage effectué au Laboratoire de Microbiologie Alimentaire et Industrielle (LMAI, ENITIAA)

Responsable scientifique : Dr Xavier Dousset

Sujet de stage : Mise au point du couplage hybridation *in situ*-cytométrie de flux pour la détection rapide et spécifique de *Carnobacterium divergens* V41.

1996 : Maîtrise de Biologie Cellulaire, Sciences et Techniques du Végétal (Dijon)

1995 : Licence de Biologie Cellulaire et Physiologie (La Roche Sur Yon)

1994 : Deug Sciences de la Nature et de la Vie (La Roche Sur Yon)

1991 : Baccalauréat D (Luçon, 85)

Expériences professionnelles antérieures-Mobilités

Septembre 2005-Aout 2006 Contrat ½ ATER (Université de la Rochelle)

Laboratoire de Biotechnologie et Chimie Bio-organique (LBCB)

Responsable scientifique : Dr Sophie Sablé

Projet : Etude du mode d'action de la microcine L

Septembre 2003-Décembre 2004 Contrat postdoctoral (INRA, Nantes)

Laboratoire des Fonctions Digestives et de Nutrition Humaine (LFDNH)

Responsable scientifique : Dr Jean-Pierre Segain

Projet : Régulation de la perméabilité intestinale par les bactéries probiotiques

Mars 2002-Février 2003 Contrat postdoctoral (Université de Caen)

Laboratoire de Microbiologie de l'Environnement (LME)

Responsable scientifique : Pr Axel Hartke

Projet : Etude du stress oxydatif chez *Lactobacillus casei* BL23, expression hétérologue de gènes impliqués dans la résistance

Octobre 2001-Janvier 2002 Ingénieur de Recherche (IFREMER, Nantes)

Laboratoire de Biochimie des Protéines et Qualité (LBPQ)

Responsable scientifique : Dr Joel Fleurence

Extraction et purification d'allergènes du thon et du cabillaud

1996 Stage volontaire de 2 mois (INRA, Dijon)

Laboratoire de Microbiologie des Sols (LMS)

Responsable scientifique : Dr Gérard Catroux

Co-cultures de *Bradyrhizobium japonicum* et *Pseudomonas* sp.

1996 Stage volontaire de 2 mois (ENSBANA, Dijon)

Laboratoire de Génie des Procédés Biotechnologiques (LGPB)

Responsable scientifique : Dr Pierre-André Maréchal

Etude de la perméabilité cellulaire chez *Saccharomyces cerevisiae*

2) ACTIVITES LIEES A L'ENSEIGNEMENT

2-1 Activité pédagogique

Mes activités d'enseignement ont débuté en 1997 au cours de mon DEA de Microbiologie Fondamentale et Appliquée et de mon doctorat de Microbiologie (1999-2001) par quelques vacances : 10 h de travaux pratiques (TP) et 2 h de travaux dirigés (TD) de Microbiologie Alimentaire, dispensés à des élèves ingénieurs au sein de l'ENITIAA (Ecole Nationale d'Ingénieurs des Techniques des Industries Agricoles et Agroalimentaire) de Nantes.

Par la suite, j'ai effectué des enseignements au CNAM de Nantes, niveau Cycle Ingénieur-Sciences et Techniques des Industries Agroalimentaires, 6 h de cours magistraux (CM) de Microbiologie Générale (structure de la cellule eucaryote, paroi bactérienne et membrane plasmique, pili et flagelles) et 20 h de TP de Microbiologie Alimentaire (stérilisation et hygiène, observation microscopique, analyse bactériologique des aliments).

En 2005-2006, j'ai été recrutée comme Attaché Temporaire d'Enseignement et de Recherche (ATER, ½ poste) à l'Université de La Rochelle. Au cours de ce contrat, j'ai réalisé 96 h d'enseignements équivalents TD, en participant à des TP de Microbiologie en Master, des CM de Physiologie Bactérienne, et des TP de Biologie Moléculaire et Biologie Cellulaire en licence.

De 2005 à 2012, j'ai également assuré des TP de Microbiologie à l'ICES (Institut Catholique d'Etudes Supérieures) de La Roche Sur Yon, en 2^{ème} année de licence, 20 h par an (suivi de croissance, identification bactérienne).

Au 1^{er} Septembre 2006, suite à l'obtention d'un concours de Maître de Conférences ouvert à l'Université de Rouen, j'ai été nommée sur le poste n°1447 (Biologie Cellulaire et Microbiologie Cellulaire, section CNU65) que j'occupe actuellement à l'IUT d'Evreux au sein du département Génie Biologique. De 2006 à 2012, j'ai effectué en moyenne 250 h eq/TD par an en présentiel. Depuis 2012 (date d'obtention de la PES, Prime d'Excellence Scientifique), j'ai réduit mon service d'enseignement pour avoir plus de temps à consacrer à mes projets de recherche.

Suivant les besoins de l'établissement, au cours de mes 9 années d'exercice à l'IUT d'Evreux, j'ai enseigné au sein de différents modules de Biologie et Microbiologie (CM, TD et TP) en 1^{ère} et 2^{ème} année de DUT Génie Biologique, dans les trois options : ABB (Analyses Biologiques et Biochimiques), IAB (Industries Alimentaires et Biologiques) et Diététique.

Lors des CM en amphithéâtre, j'assure mes cours grâce à un support powerpoint qui permet d'illustrer et d'animer les mécanismes biologiques que j'enseigne. Les étudiants reçoivent les diapositives à la fin de chaque chapitre ainsi qu'une liste de sites internet en lien avec le cours afin qu'ils puissent réviser et approfondir leurs connaissances. Pendant les TD, j'aborde des notions théoriques, puis je fais effectuer aux étudiants des analyses de documents (figures, textes en français ou en anglais, issus d'expériences scientifiques ou présentant de nouvelles technologies). Les TP que j'enseigne servent à acquérir les bonnes pratiques de laboratoire en Biologie et Microbiologie. Ils permettent notamment de montrer à chaque étudiant comment manipuler stérilement en Biologie cellulaire et Microbiologie et comment réaliser certains dosages biologiques.

En plus de ces enseignements présentiels au sein du DUT Génie biologique, j'encadre chaque année les stages de 5 étudiants de l'option IAB, ABB ou Diététique. J'effectue des visites au sein de la structure d'accueil et/ou je fais un suivi par téléphone, pour m'assurer du bon déroulement des stages et entretenir un lien avec les industriels. Je participe aussi à l'encadrement des projets tutorés et projets personnels et professionnels (PPP) en 1^{ère} et 2^{ème} année de DUT, avec pour objectif d'aider les étudiants à découvrir les métiers en lien avec la spécialité Génie Biologique, les orienter dans leur parcours universitaire et/ou leur insertion professionnelle. Dans le cadre du plan réussite en licence de l'Université de Rouen, j'ai aussi été amenée à être la tutrice de plusieurs étudiants issus de baccalauréats technologiques afin de favoriser leur intégration au sein de la formation, et aider à leur apprentissage grâce à des entretiens individualisés et conseils pour la révision des cours.

Depuis 2013, j'interviens également au sein de la formation Master 2 Biosciences, Microbiologie, de l'Université de Rouen. J'assure 4 h de CM et analyses d'articles scientifiques sur mes thèmes de recherche : les interactions bactéries-intestin et le microbiote intestinal. Ceci apporte aux étudiants des connaissances pointues sur les bactéries intestinales, et constitue pour eux une première approche de la recherche scientifique avant leur immersion en stage.

Depuis septembre 2015, je participe aussi à des enseignements au sein de la licence professionnelle Bioindustries et Biotechnologies, Parcours Toxicologie et Gestion des risques toxiques, de l'université de Rouen-IUT d'Evreux. J'effectue dans cette formation 4 h de CM et 8 h de TP sur les risques microbiologiques, ainsi que 3 h de CM/TD de compléments scientifiques sur la physiologie digestive, hépatique et rénale.

2-2 Responsabilités collectives

L'IUT d'Evreux et le DUT Génie Biologique (GB) ont été créés en 1985. A l'époque, une seule option de DUT GB existait, l'option Industries Alimentaires et Biologiques (IAB). L'option Diététique a ensuite vu le jour en 1998. En 2009, le conseil de département GB et son équipe pédagogique propose d'ouvrir une troisième option du DUT GB, l'option Analyses Biologiques et Biochimiques (ABB), afin d'élargir le panel de formations en adéquation avec les demandes des industriels de la pharmaceutique et cosmétique, et d'augmenter l'attractivité de l'IUT d'Evreux. J'ai pris en charge la rédaction de la demande d'ouverture de l'option ABB qui a été soumise à l'avis du conseil de l'IUT d'Evreux, puis au CEVU (Conseil des Etudes et de la Vie Etudiante) et au CA (Conseil d'Administration) de l'Université de Rouen, au LEEM (les entreprises du médicament), et transmise au Ministère de l'Enseignement Supérieur et de la Recherche. La demande a été acceptée pour une ouverture effective en Septembre 2010. J'ai donc pris en toute logique, la responsabilité pédagogique de cette nouvelle option du DUT GB de Septembre 2010 à Aout 2013. La première de mes tâches a été de constituer l'équipe pédagogique, à partir des ressources en personnels enseignants (professeurs certifiés et professeurs agrégés) et enseignant-chercheurs (maitre de conférences et professeurs d'université), disponibles à l'IUT d'Evreux pour les cours de Physiologie, Biologie Cellulaire, Biologie Moléculaire, Biochimie, Microbiologie, Pharmacologie, Toxicologie et Statistiques, mais aussi en faisant appel à des intervenants extérieurs (environ 20), experts de leur domaine d'intervention pour les enseignements de Bactériologie Médicale, Parasitologie, Virologie, Hématologie, Droit du Travail et Législation Cosmétique... En DUT ABB, près de 40% des enseignements en présentiel sont effectués par des vacataires extérieurs à l'IUT afin de permettre aux étudiants, d'avoir un premier contact avec des industriels de leur secteur d'activité, et leur apprendre ce qui se fait réellement « sur le terrain ».

Outre le recrutement des vacataires, mon rôle en tant que responsable pédagogique a été de mettre en place les emplois du temps (CM, TD et 2 groupes de TP), les réajuster en fonction des besoins et imprévus, les saisir sur le logiciel ADE qui permet aussi de réserver les salles (amphithéâtres, salles de TD, salles de TP), et prévoir les commandes pour les TP en créant un lien entre les enseignants vacataires et les techniciens de laboratoire. J'ai également participé aux réflexions sur le contenu des UE et modules enseignés avec l'ensemble de l'équipe pédagogique, collecté les notes de chaque module, et préparé les conseils de semestre et jury. Enfin, j'ai réalisé un suivi des étudiants avec entretien individuel pour les conseiller en cas de difficultés, ou pour les aider à murir leur projet professionnel et/ou leur poursuite d'études à l'université ou en école d'ingénieur.

La tâche de responsable pédagogique du DUT ABB étant très chronophage, et ayant obtenu la Prime d'Excellence Scientifique (PES) en Octobre 2012, j'ai décidé d'arrêter cette responsabilité à la rentrée universitaire suivante (Septembre 2013) afin de pouvoir me consacrer davantage à mes activités de recherche.

Actuellement, les responsabilités collectives que je continue à exercer à l'IUT dans le cadre de l'enseignement sont :

- la responsabilité du module Cultures cellulaires-Méthodes alternatives, DUT GB2 ABB
- la responsabilité du module Microbiologie Médicale, DUT GB2 ABB
- la responsabilité du module Physiologie-Pharmacologie, DUT GB1 préoption ABB
- la responsabilité du module Nutrition-Physiologie, DUT GB1 préoption Diététique

Pour ces modules, je veille à ce que le contenu enseigné par les différents intervenants soit conforme au Plan Pédagogique National (PPN GB 2013) du DUT GB et j'assure la collecte des notes et le calcul des moyennes des étudiants. Ces notes sont présentées et validées lors de la commission pédagogique du DUT GB.

Chaque année, je participe aux différents forums et journées portes ouvertes qui sont organisés par l'établissement afin de promouvoir l'IUT et le département GB, renseigner les futurs étudiants et leurs parents sur les trois parcours possibles IAB, ABB et Diététique du DUT GB de l'IUT d'Evreux, et les orienter dans leur choix pour l'inscription sur le site internet admission postbac. <http://www.admission-postbac.fr/>

Depuis 2013, je suis assistante de prévention Biologie au sein de l'IUT. Je dois m'assurer que les TP de Biologie et Microbiologie effectués dans l'établissement comportent le minimum de risques biologiques pour les étudiants (travail sur des souches non pathogènes ou avec virulence atténuée, non-dangérosité des échantillons cliniques utilisés pour l'option ABB). Je participe à la mise à jour du classeur de sécurité, à la rédaction du Document Unique et aux réunions de la section locale HSCTE (Hygiène-Sécurité-Conditions de Travail-Environnement).

Je fais également partie du groupe de travail PPMS (Plan Particulier de Mise en Sûreté) de l'IUT, qui a pour objectif d'établir les règles de confinement au sein de l'établissement, pour mettre en sécurité toutes les personnes présentes dans les locaux en cas de survenue d'un accident majeur externe à l'IUT (tempête, nuage toxique, accident nucléaire...).

Enfin, je suis représentante universitaire au sein de la commission pédagogique et de la commission d'attribution des crédits (CAC) de l'Institut de Formation en Soins Infirmiers d'Evreux (IFSIE) depuis Mars 2011.

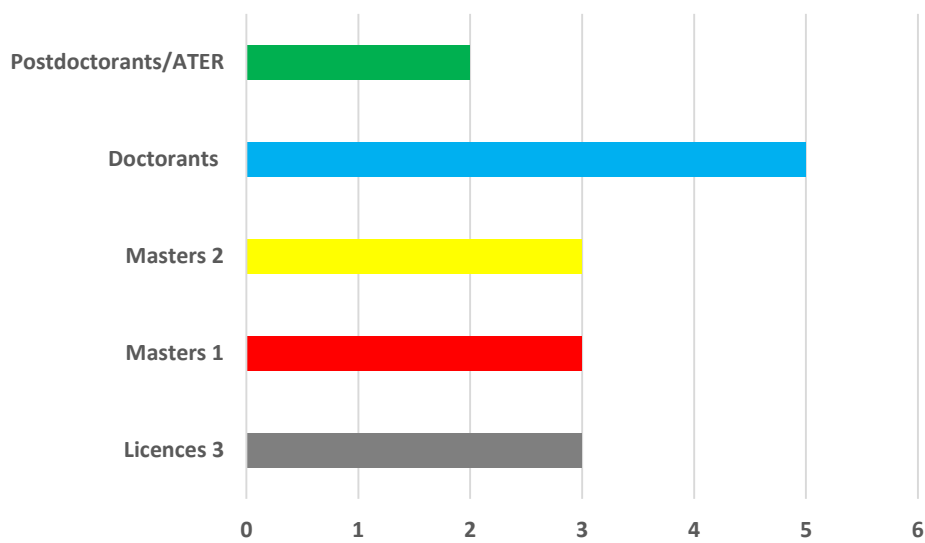


Figure 1 : Nombre d'étudiants encadrés ou co-encadrés depuis septembre 2006, date de mon recrutement comme maître de conférences à l'université de Rouen/IUT d'Evreux.

3) ACTIVITES D'ENCADREMENTS ET DE FORMATION

Le nombre d'étudiants que j'ai encadrés au cours de mes activités de recherche est présenté ci-dessous et sur la **Figure 1**. Ma participation à l'encadrement des postdoctorants, ATER et doctorants est précisée en pourcentage.

Postdoctorants et ATER

Ziad Alnabhani (2013)

« Etude des mécanismes de signalisation utilisés par *Pseudomonas fluorescens* pour altérer la barrière intestinale, implication de NOD2 »

Taux d'encadrement 30%

En collaboration avec le Pr Jean-Pierre Hugot et le Dr Frédérick Barreau
INSERM U843, Inflammation intestinale pathologique de l'enfant

Amar Madi (2010-2012)

« Effet de la β -defensine 2 sur la virulence de *Pseudomonas fluorescens* »

Taux d'encadrement 100%

½ ATER LMSM

Doctorants du laboratoire LMSM

Kelly Biaggini (2012-2015)

« Rôle du dialogue entre le système enteroendocrinien et la microflore intestinale dans les réactions inflammatoires de l'intestin »

Co-responsabilité de thèse avec le Pr Marc Feuilloley (LMSM) et le Pr Pierre Déchelotte, INSERM U1073, Nutrition, inflammation et dysfonction de l'axe intestin-cerveau

Puis co-direction de thèse (75%) avec le Pr Pierre Déchelotte depuis 2013.

Soutenance prévue le 14 décembre 2015.

Charlène Leneveu (2010-2014)

« Virulence et adaptation à l'environnement de souches de *Pseudomonas fluorescens* et *Pseudomonas mosselii*. Contribution à l'étude des conditions d'expression et des fonctions de la protéine TSPO chez *P. fluorescens* »

Co-direction de thèse (50%) avec le Pr Sylvie Chevalier (LMSM)

Thèse soutenue le 1^{er} septembre 2014.

Amar Madi (2007-2010)

« Interaction de *Pseudomonas fluorescens* avec l'épithélium intestinal. Rôle dans l'inflammation et la perméabilité intestinale et implication potentielle dans la maladie de Crohn »

Co-responsabilité de thèse (50%) avec le Pr Marc Feuilloley (LMSM)

Thèse soutenue le 8 décembre 2010.

Collaboration à des thèses extérieures au LMSM

Soumaya Messaoudi (Thèse soutenue en 2012)

« *Lactobacillus salivarius* SMXD51, isolé du caecum du poulet tunisien : caractérisation de sa bactériocine anti-*Campylobacter* et de son potentiel probiotique »

Collaboration avec le Pr Xavier Dousset, UMR SECALIM INRA ONIRIS 1014, Nantes

2 publications en co-auteur.

Messaoudi S, Manai M, Kergourlay G, Prévost H, **Connil N**, Chobert JM, Dousset X (2013). *Lactobacillus salivarius*: bacteriocin and probiotic activity. Food Microbiol. 36(2):296-304. (IF₂₀₁₃=**3,374**)

Messaoudi S, Madi A, Prévost H, Feuilleley M, Manai M, Dousset X, **Connil N** (2012). *In vitro* evaluation of the probiotic potential of *Lactobacillus salivarius* SMXD51. Anaerobe. 18(6):584-589. (IF₂₀₁₂=**2,022**)

Yanath Belguesmia (Thèse soutenue en 2010)

« Purification et caractérisation du mode d'action et de l'innocuité de l'entéroisine S37, une bactériocine produite par *Enterococcus faecalis* S37 »

Collaboration avec le Dr Djamel Drider, UMR SECALIM INRA ONIRIS 1014, Nantes

1 publication en co-auteur.

Belguesmia Y, Madi A, Sperandio D, Merieau A, Feuilleley M, Prévost H, Drider D, **Connil N** (2011). Growing insights into the safety of bacteriocins: the case of enterocin S37. Res Microbiol. 162(2):159-163. (IF₂₀₁₁=**2,763**)

Natacha Morin (Thèse soutenue en 2008)

« Etude du mécanisme d'action et de la régulation de la production de la microisine L »

Collaboration avec le Dr Sophie Sablé et Dr Isabelle Lanneluc,

Laboratoire de biotechnologies et chimie bio-organique FRE2766 CNRS

Université de la Rochelle

1 publication en co-auteur.

Morin N, Lanneluc I, **Connil N**, Cottenneau M, Pons AM, Sablé S (2011). Mechanism of bactericidal activity of microisin L in *Escherichia coli* and *Salmonella enterica*. Antimicrob Agents Chemother. 55(3):997-1007. (IF₂₀₁₁=**4,841**)

Etudiants de niveau master 2 et ingénieur (Stages de 6 mois)

Valérie Borrel (2015)

« Etude de l'effet pro-virulent de l'épinéphrine et de la substance P chez *Enterococcus faecalis* » Master 2 Biosciences, Microbiologie, Université de Rouen

Ziad Alnabhani (2010)

« Modulation de la virulence chez *Pseudomonas* par des facteurs eucaryotes intestinaux »

Master 2 Microbiologie Fondamentale et Appliquée, Université de Rennes

Amar Madi (2008)

« Maladie de Crohn : implication potentielle de *Pseudomonas fluorescens* »

Master 2 Biosciences, Microbiologie, Université de Rouen

Anne Brillet (2001)

« Sélection et caractérisation d'un mutant tyrosine décarboxylase négatif de *Carnobacterium divergens* V41 producteur d'une bactériocine active contre *Listeria monocytogenes* »
DEA Microbiologie Fondamentale et Appliquée, Université de Rennes

Léon Plissoneau (2000)

« Etude de la croissance, des activités enzymatiques et inhibitrices de *Carnobacterium divergens* V41. Implantation de la souche sur le saumon fumé »
Ingénieur CNAM, Nantes

Etudiants de niveau licence et master 1 (Stages de 2 à 3 mois)**Alexis Dupuis (2014)**

« Analyse du microbiote intestinal d'un modèle murin d'anorexie (souris ABA) »
Master 1, Biosciences, Microbiologie, Université de Rouen

Siham Bouhelbane (2014)

« Identification bactérienne à partir de contenus caecaux de souris anorexiques »
Licence 3, Biologie, Université de Rouen

Stéphanie Mwanja (2012)

« Effet de la β -défensine 2 (hBD-2) sur la virulence de *Pseudomonas fluorescens* »
Licence 3, Ingénierie de la Santé, Université de Rouen

Nassima Selmani (2011)

« Production de β -défensine 2 par les cellules Caco-2/TC7 après infection par *Pseudomonas* et effet de ce peptide sur la virulence bactérienne »
Master 1, Microbiologie Fondamentale et Appliquée, Université de Rennes

Faten Gazdallah (2008)

« Interaction de *Pseudomonas fluorescens* avec les cellules intestinales différenciées ».
Master 1, Ingénierie de la Santé, Université de Rouen

Claudie Laurent (2007)

« Effet de la guanyline sur la virulence de *Pseudomonas* »
Licence 3, Ingénierie de la Santé, Université de Rouen

Said Ihammouine (2006)

« Production et purification de la microcine L produite par *Escherichia coli* »
Licence 3, Sciences de la Vie, Biochimie, Université de la Rochelle

Etudiant de niveau BTS (Stage de 6 semaines)**Constant Guillet (2015)**

« Effet de l'épinéphrine sur la formation de biofilms chez *Pseudomonas aeruginosa* »
BTS Bioanalyses et contrôles, Lycée Léopold Sédar Senghor, Evreux

Etudiant ERASMUS (Stage de 2 mois)

Rikki Knowlton (2014)

« Modulation de la cytotoxicité d'*Enterococcus faecalis* V583 par la substance P »
Licence 1, Université de Portsmouth, UK

4) FONCTIONS ADMINISTRATIVES EN LIEN AVEC LA RECHERCHE ET PARTICIPATION A DES RESEAUX ET CONTRATS

- Responsable du microscope confocal au LMSM
- Membre de la commission CCSE 64-65 de l'Université de Rouen depuis 2009 et élue à la commission CCSE restreinte en 2010, pour le recrutement d'ATER et titularisation
- Membre du GRR CBS Réseau Sésa
- Membre de l'IRIB (Institute for Research and Innovation in Biomedecine)
- Membre de la Fédération de Microbiologie du Grand Ouest
- Demandes de financement ANR
 - Dépôt du projet FluoCrohn en 2009 (non retenu)
 - Participation au Projet Microghrelin déposé en 2014 par le Pr Serguei Fetissov, INSERM UMR1073 Nutrition, inflammation et dysfonction de l'axe intestin-cerveau, Université de Rouen (non retenu)
- Participation à la rédaction du projet COS-SESA (GRR FEDER-FUI), partie étude Probiot
- Obtention d'un financement de l'IRIB pour l'analyse du microbiote de souris anorexiques par Maldi-Biotyper
- Obtention d'une Bourse Régionale Industrielle (BRI) pour Amar Madi et d'allocations régionales pour Charlène Leneveu et Kelly Biaggini
- Recherche d'un financement industriel ou public pour une thèse qui démarrerait en Octobre 2016

5) COLLABORATIONS, EXPERTISES, EVALUATIONS

Collaborations nationales

-UMR SECALIM INRA ONIRIS 1014, Nantes

Pr Hervé Prévost, Pr Xavier Dousset, Dr Marie-France Pilet

-UMR 1319 INRA MICALIS, Jouy en Josas

Dr Joël Doré, Dr Hervé Blottière, Dr Pascale Serror, Dr Cristel Archambaud, Dr Lionel Rigottier-Gois

-INSERM U843, Inflammation intestinale pathologique de l'enfant, Paris

Dr Jean-Pierre Hugot, Dr Frédérick Barreau

-Institut Pasteur, Laboratoire microenvironnement et immunité, Paris

Dr Ziad Alnabhani

-INSERM U1073, Nutrition, inflammation et dysfonction de l'axe intestin-cerveau, Rouen

Pr Pierre Déchelotte, Pr Serguei Fetissov, Dr Moïse Coeffier, Dr Rachel Marion-Letellier

-EA7394, Institut Régional de Recherche en Agro-alimentaire et Biotechnologie "Charles VIOLLETTE", Lille et Boulogne Sur Mer

Pr Djamel Drider, Dr Maryse Bonnin-Jusserand

-UMR-CNRS 8520, Institut d'électronique, de microelectronique et de nanotechnologie, Lille

Pr Sabine Szunerits

-ANSES, Boulogne Sur Mer

Dr Guillaume Duflos

-UMR INRA 1280-PhAN, Physiologie des adaptations nutritionnelles, Nantes

Dr Jean-Pierre Segain, Dr Pierre De Coppet

-EA 4655 – UR Risques microbiens (U2RM), Caen

Pr Alain Rincé

Collaborations internationales

-Université de Tunis, Tunisie, (Pr Mohammed Manai)

-Université de São Paulo, Brésil (Dr Svetoslav Todorov)

-McGill University Health Centre, Montréal, Canada (Pr Vassilios Papadopoulos)

-Université de Sofia (Dr Petia Kabadjova), projet de mise en place d'un programme ERASMUS

-Université Federico 2, Naples (Dr Alessandra Pollice), dépôt d'un projet Galileo en 2014

Participation à des commissions de recrutement et jurys de thèse

-Membre du comité de sélection du poste de Maître de Conférences 66MCF0586, INSERM U1073, Université de Rouen, Mai 2015

-Membre du comité de sélection du poste de Maître de Conférences MC-19671, Oniris, Nantes, Juin 2012

-Membre du comité de sélection du poste de Maître de Conférences 64MCF1081, U2RM, Université de Caen, Mai 2012

-Membre du comité de sélection du poste de Maître de Conférences 64MCF1474, LMSM, Université de Rouen, Mai 2011

-Membre examinateur dans le jury de la thèse de Yanath Belguesmia, soutenue le 25 Octobre 2010, Université de Nantes

-Participation aux comités de thèses d'Ahmed Al Taya et Alaa Al-Serah, 22 Septembre 2014, Université de Lille

Expertises (reviewer) pour des revues internationales

African Journal of Microbiology Research

Anaerobe

Archives of Microbiology

British Microbiology Research Journal

Canadian Journal of Microbiology

Current Microbiology

Probiotics and Antimicrobial Proteins

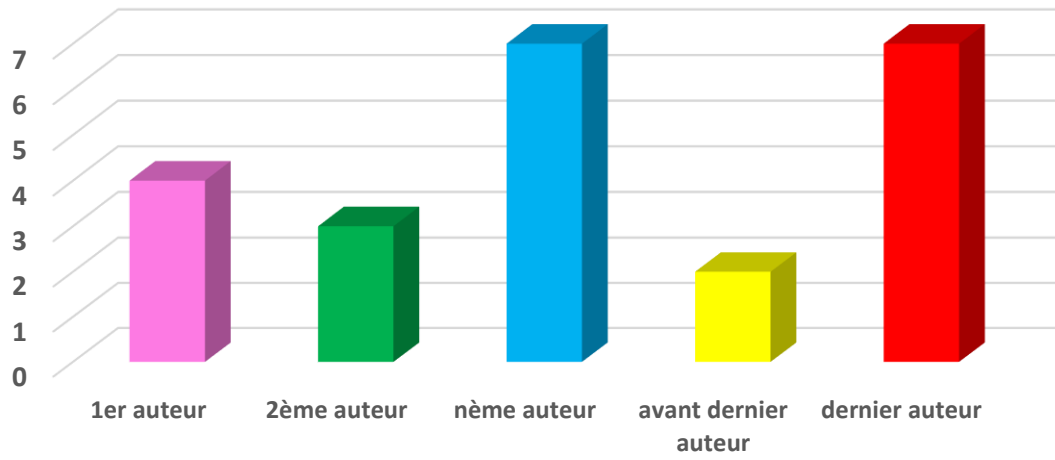
Veterinary Microbioloy

Distinctions et évaluations

2003 : Obtention du prix Jeune Chercheur (Institut Français de la Nutrition)

2012-2016 : Obtention de la PES (Prime d'Excellence Scientifique)

A



B

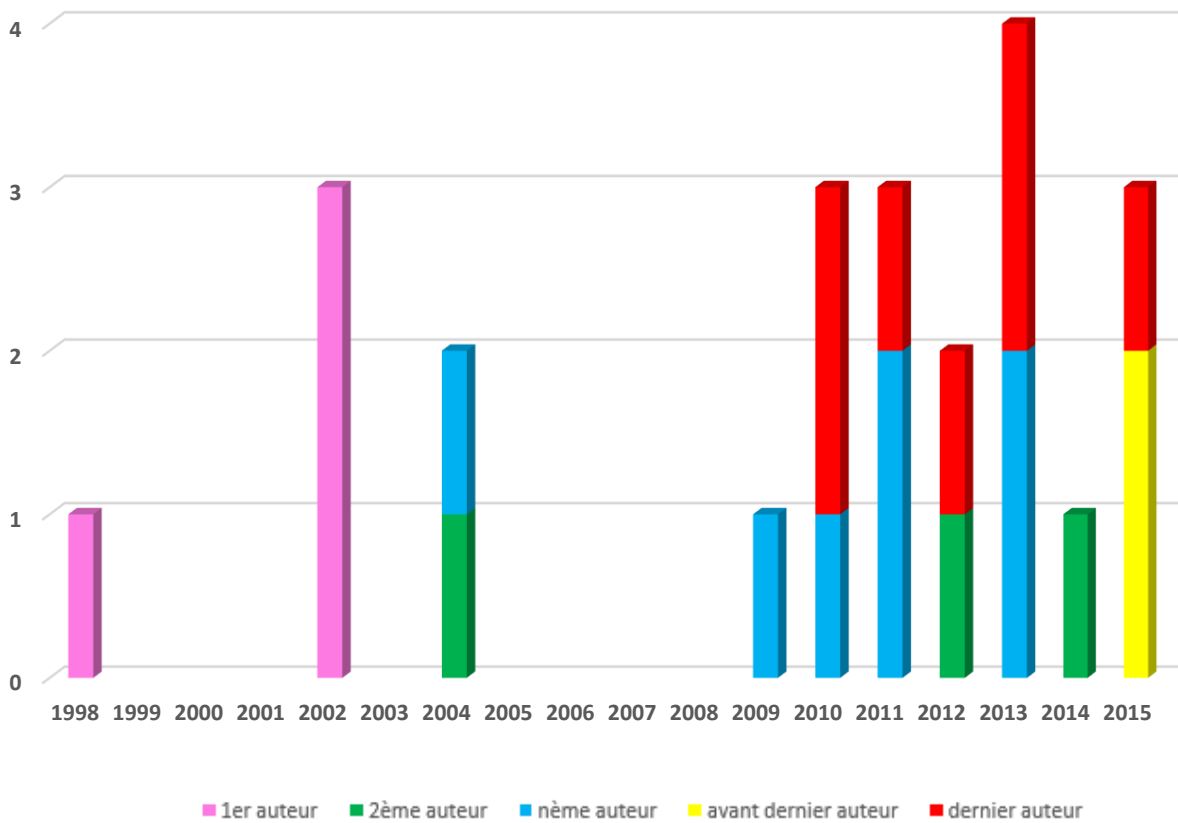


Figure 2 : Bilan des publications dans des revues à comité de lecture par position d'auteur (A) et détaillé par année (B) (nème auteur= de la 3^{ème} position à l'avant-avant dernière position)

6) PRODUCTION SCIENTIFIQUE

6-1 Publications dans des revues avec comité de lecture (TOTAL=23)

J'ai publié 23 articles dont 17 depuis mon recrutement au concours de maitre de conférences du LMSM (soit une moyenne de 1,89 article par an sur la période 2006-2015). Je possède 7 publications en dernier auteur, 2 en avant-dernier auteur et 4 en 1^{er} auteur (Figure 2). Un bilan des publications des doctorants et masters 2, que j'ai encadrés au LMSM, est également présenté sur la Figure 3.

Leneveu-Jenvrin C, Bouffartigues E, Maillot O, Cornelis P, Feuilloley M, **Connil N**, Chevalier S (2015). Expression of the translocator protein (TSPO) from *Pseudomonas fluorescens* Pf0-1 requires the stress regulatory sigma factors AlgU and RpoH. *Front Microbiol.* 6:1023. (IF₂₀₁₄=3,989)

Biaggini K, Barbey C, Borrel V, Feuilloley M, Déchelotte P, **Connil N** (2015). The pathogenic potential of *Pseudomonas fluorescens* MFN1032 on enterocytes can be modulated by serotonin, substance P and epinephrine. *Arch Microbiol.* 197(8):983-990. (IF₂₀₁₄=1,667)

Alnabhani Z, Montcuquet N, Biaggini K, Dussailant M, Roy M, Ogier-Denis E, Madi A, Jallane A, Feuilloley M, Hugot JP, **Connil N**, Barreau F (2015). *Pseudomonas fluorescens* alters intestinal barrier function by modulating IL-1 β expression through hematopoietic NOD2 signaling. *Inflamm Bowel Dis.* 21(3):543-555. (IF₂₀₁₄=5,475)

Leneveu-Jenvrin C, **Connil N**, Bouffartigues E, Papadopoulos V, Feuilloley MG, Chevalier S (2014). Structure-to-function relationships of bacterial translocator protein (TSPO): a focus on *Pseudomonas*. *Front Microbiol.* 5:631. (IF₂₀₁₄=3,989)

Leneveu-Jenvrin C, Madi A, Bouffartigues E, Biaggini K, Feuilloley M, Chevalier S, **Connil N** (2013). Cytotoxicity and inflammatory potential of two *Pseudomonas mosselii* strains isolated from clinical samples of hospitalized patients. *BMC Microbiol.* 13:123. (IF₂₀₁₃=2,976)

Madi A, Alnabhani Z, Leneveu C, Mijouin L, Feuilloley M, **Connil N** (2013). *Pseudomonas fluorescens* can induce and divert the human β -defensin-2 secretion in intestinal epithelial cells to enhance its virulence. *Arch Microbiol.* 195(3):189-195. (IF₂₀₁₃=1,861)

Gicquel G, Bouffartigues E, Bains M, Oxaran V, Rosay T, Lesouhaitier O, **Connil N**, Bazire A, Maillot O, Bénard M, Cornelis P, Hancock RE, Dufour A, Feuilloley MG, Orange N, Déziel E, Chevalier S (2013). The extra-cytoplasmic function sigma factor sigX modulates biofilm and virulence-related properties in *Pseudomonas aeruginosa*. *PLoS One.* 8(11):e80407. (IF₂₀₁₃=3,534)

Messaoudi S, Manai M, Kergourlay G, Prévost H, **Connil N**, Chobert JM, Dousset X (2013). *Lactobacillus salivarius*: bacteriocin and probiotic activity. *Food Microbiol.* 36(2):296-304. (IF₂₀₁₃=3,374)

Messaoudi S, Madi A, Prévost H, Feuilloley M, Manai M, Dousset X, **Connil N** (2012). *In vitro* evaluation of the probiotic potential of *Lactobacillus salivarius* SMXD51. *Anaerobe*. 18(6):584-589. **(IF₂₀₁₂=2,022)**

Reffuveille F, **Connil N**, Sanguinetti M, Posteraro B, Chevalier S, Auffray Y, Rince A. (2012). Involvement of peptidylprolyl cis/trans isomerases in *Enterococcus faecalis* virulence. *Infect Immun*. 80(5):1728-1735. **(IF₂₀₁₂=4,074)**

Belguesmia Y, Madi A, Sperandio D, Merieau A, Feuilloley M, Prévost H, Drider D, **Connil N** (2011). Growing insights into the safety of bacteriocins: the case of enterocin S37. *Res Microbiol*. 162(2):159-163. **(IF₂₀₁₁=2,763)**

Fito-Boncompte L, Chapalain A, Bouffartigues E, Chaker H, Lesouhaitier O, Gicquel G, Bazire A, Madi A, **Connil N**, Véron W, Taupin L, Toussaint B, Cornelis P, Wei Q, Shioya K, Déziel E, Feuilloley MG, Orange N, Dufour A, Chevalier S (2011). Full virulence of *Pseudomonas aeruginosa* requires OprF. *Infect Immun*. 79(3):1176-1186. **(IF₂₀₁₁=4,165)**

Morin N, Lanneluc I, **Connil N**, Cottenceau M, Pons AM, Sablé S (2011). Mechanism of bactericidal activity of microcin L in *Escherichia coli* and *Salmonella enterica*. *Antimicrob Agents Chemother*. 55(3):997-1007. **(IF₂₀₁₁=4,841)**

Madi A, Svinareff P, Orange N, Feuilloley MG, **Connil N** (2010). *Pseudomonas fluorescens* alters epithelial permeability and translocates across Caco-2/TC7 intestinal cells. *Gut Pathog*. 2(1):16. **(IF₂₀₁₀=2,109)**

Madi A, Lakhdari O, Blottière HM, Guyard-Nicodème M, Le Roux K, Groboillot A, Svinareff P, Doré J, Orange N, Feuilloley MG, **Connil N** (2010). The clinical *Pseudomonas fluorescens* MFN1032 strain exerts a cytotoxic effect on epithelial intestinal cells and induces Interleukin-8 via the AP-1 signaling pathway. *BMC Microbiol*. 10:215. **(IF₂₀₁₀=2,96)**

Sperandio D, Rossignol G, Guerillon J, **Connil N**, Orange N, Feuilloley MG, Merieau A (2010). Cell-associated hemolysis activity in the clinical strain of *Pseudomonas fluorescens* MFN1032. *BMC Microbiol*. 10:124. **(IF₂₀₁₀=2,96)**

Lesouhaitier O, Veron W, Chapalain A, Madi A, Blier AS, Dagorn A, **Connil N**, Chevalier S, Orange N, Feuilloley M (2009). Gram-negative bacterial sensors for eukaryotic signal molecules. *Sensors (Basel)*. 9(9):6967-6990. **(IF₂₀₀₉=1,821)**

Lebois M, **Connil N**, Onno B, Prévost H, Dousset X (2004). Effects of divercin V41 combined to NaCl content, phenol (liquid smoke) concentration and pH on *Listeria monocytogenes* ScottA growth in BHI broth by an experimental design approach. *J Appl Microbiol*. 96(5):931-937. **(IF₂₀₀₄=1,835)**

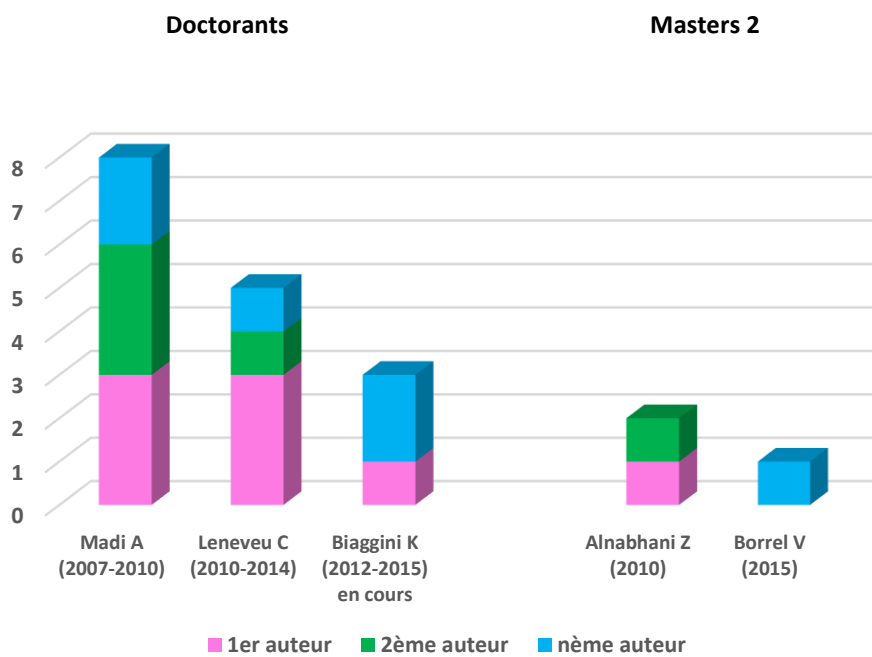


Figure 3 : Bilan des publications par doctorants et étudiants en master 2, liées à des travaux que j’ai encadrés au LMSM (nème auteur= de la 3^{ème} position à l’avant-avant dernière position)

Richard C, Leroi F, Brillet-Viel A, Rachman C, **Connil N**, Drider D, Pilet MF, Onno B, Dousset X, Prevost H (2004). Maîtrise du développement de *Listeria monocytogenes* dans le saumon fumé : intérêt de la biopréservation par des bactéries lactiques. Lait. 84 (1-2), 135-144. **(IF₂₀₀₄=1,043)**

Connil N, Le Breton Y, Dousset X, Auffray Y, Rincé A, Prévost H (2002). Identification of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine production. Appl Environ Microbiol. 68(7):3537-3544. **(IF₂₀₀₂=3,691)**

Connil N, Prévost H, Dousset X (2002). Production of biogenic amines and divercin V41 in cold smoked salmon inoculated with *Carnobacterium divergens* V41, and specific detection of this strain by multiplex-PCR. J Appl Microbiol. 92(4):611-617. **(IF₂₀₀₂=1,819)**

Connil N, Plissoneau L, Onno B, Pilet MF, Prévost H, Dousset X (2002). Growth of *Carnobacterium divergens* V41 and production of biogenic amines and divercin V41 in sterile cold-smoked salmon extract at varying temperatures, NaCl levels, and glucose concentrations. J Food Prot. 65(2):333-338. **(IF₂₀₀₂=1,686)**

Connil N, Dousset X, Onno B, Pilet MF, Breuil MF, Montel MC (1998). Enumeration of *Carnobacterium divergens* V41, *Carnobacterium piscicola* V1 and *Lactobacillus brevis* LB62 by *in situ* hybridization-flow cytometry. Lett Appl Microbiol. 27(5):302-306. **(IF₂₀₁₄=1,749)**

6-2 Communications dans des congrès et séminaires (TOTAL=31)

Biaggini K, Barbey C, Feuilloley M, Déchelotte P, **Connil N** (2015). The pathogenic potential of *Pseudomonas fluorescens* can be modulated by serotonin, substance P and epinephrine. ECCMID, 25th European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, 25-28 Avril 2015.

Biaggini K, Feuilloley M, Déchelotte P, **Connil N** (2015). Les catécholamines modulent la cytotoxicité, l'adhésion/invasion et la translocation des bactéries intestinales. CECED, Nantes, 26-27 Mars 2015.

Connil N (2014). Effet de molécules eucaryotes sur la virulence de *Pseudomonas fluorescens* et *Enterococcus faecalis*. Séminaire du Pôle Risques- Equipe CPE Commensalisme et Pathogénie des Entérocoques, MICALIS- INRA de Jouy en Josas, le 24 Septembre 2014.

Biaggini K, **Connil N**, Coëffier M, Déchelotte P, Feuilloley M (2014). The virulence behavior of *Pseudomonas fluorescens* MFN1032 can be modulated by gut secretion. Congrès de l'IRIB, Caugé, 20 Juin 2014.

Biaggini K, **Connil N**, Coëffier M, Déchelotte P, Feuilloley M (2014). The virulence behavior of *Pseudomonas* can be modulated by gut secretion. 17^{ième} Journée de l'EdNBISE, Le Havre, 10 Avril 2014.

Biaggini K, Coëffier M, Déchelotte P, Feuilloley M, **Connil N** (2014). Effet de la substance P, de la sérotonine et de l'épinephrine sur la croissance et la virulence de bactéries intestinales. CECED, Journées Francophones d'Hépatogastroentérologie et d'Oncologie Digestive, Paris, 20-23 Mars 2014.

Leneveu-Jenvrin C, Madi A, Bouffartigues E, Biaggini K, Orange N, Feuilloley MGJ, Chevalier S, **Connil N** (2013). Cytotoxicity and inflammatory potential of two *Pseudomonas mosselii* strains isolated from clinical samples of hospitalized patients 14th international conference on *Pseudomonas*, Lausanne, Switzerland, 7-11 Septembre 2013.

Biaggini K, **Connil N**, Coëffier M, Déchelotte P, Feuilloley M (2013). Effect of substance P, epinephrin and serotonin on growth and virulence of intestinal bacteria. 16^{ème} Journée de l'Ecole Doctorale EdNBISE, Caen, France, 11-12 Juillet 2013.

Alnabhani Z, Madi A, Dussaillant M, Feuilloley M, Hugot J-P, **Connil N**, Barreau F (2013). *Pseudomonas fluorescens* increases the paracellular permeability of Peyer's patches and ileal mucosa by mechanisms dependent of Nod2 and IL1R pathways. Digestive Disease Week, Orlando, USA, 18-21 Mai 2013.

Poster publié dans Gastroenterology, Volume 144, Issue 5, Supplement 1, May 2013, Page S-825

Hillion M, Mijouin L, Dagorn A, Leneveu C, Blier AS, Madi A, Jaouen T, Rosay T, Biaggini K, **Connil N**, Poc C, Groboillot A, Hulen C, Lomri N, Lesouhaitier O, Orange N, Feuilloley MGJ (2013). Another paradigm in antibacterial treatments : Why a diplomatic approach should be more efficient than weapons of mass destruction. ESF-EMBO Symposium Bacterial Networks, Pultusk, Poland, 16-21 Mars 2013.

Madi A, Alnabhani Z, Leneveu C, Mijouin L, Feuilloley M, **Connil N** (2013). Modulation de la virulence de *Pseudomonas fluorescens* par HBD-2. Congrès de la SFM (Société Française de Microbiologie), Lille, 7-8 Février 2013.

Messaoudi S, **Connil N**, Prévost H, Chobert JM, Federighi M, Manai M, Dousset X (2013). *Lactobacillus salivarius* SMXD51, isolé du caecum du poulet : caractérisation de sa bactériocine anti-*Campylobacter* et de son potentiel probiotique, Congrès de la SFM (Société Française de Microbiologie), Lille, 7-8 Février 2013.

Messaoudi S, Madi A, Feuilleley M, Prévost H, Chobert JM, Manai M, **Connil N**, Dousset X (2012). Etude du potentiel probiotique de *Lactobacillus salivarius* SMXD51, souche isolée de caecum de poulet à activité anti-*Campylobacter*. Journées des microbiologistes de l'INRA, L'Isle-sur-la-Sorgue, 13-15 Novembre 2012.

Hillion M, Mijouin L, Dagorn A, Rosay T, Madi A, Jaouen T, **Connil N**, Poc C, Groboillot A, Lesouhaitier O, Orange N, Chevalier S, Feuilleley MGJ (2012). *Pseudomonas fluorescens*, a member of the gut-brain-skin axis. 1ère journée scientifique de l'Institut de Recherche et d'Innovation Biomedicale (IRIB), Rouen (France), 1er Juin 2012.

Leneveu C, Madi A, Bouffartigues E, Orange N, Feuilleley MGJ, Chevalier S, **Connil N** (2012). Cytotoxicity and inflammatory potential of three *Pseudomonas mosselii* clinical strains on Caco2/TC7 intestinal cells. SGM Spring Conference 2012. DU16 Hospital-acquired infections, Dublin, Irlande, 28-29 Mars 2012.

Sperandio D, Rossignol G, Guerillon J, **Connil N**, Feuilleley M, Orange N, Merieau A (2010). Activité hémolytique cellules-associées chez une souche clinique de *Pseudomonas fluorescens* MFN1032. Congrès de la SFM (Société Française de Microbiologie), Marseille, 2-4 Juin 2010.

Svinareff P, Orange N, Feuilleley MGJ, **Connil N** (2010). *Pseudomonas fluorescens* can increase the permeability of Caco2/TC7 and modify the actin cytoskeleton. ECCMID, 20th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, 10-13 Avril 2010.

Madi A, **Connil N**, Svinareff P, Orange N, Feuilleley MGJ (2009). *Pseudomonas fluorescens* modulates permeability and translocates across Caco-2/TC7 polarized epithelial monolayers. Gastro 2009, UEGW/WCOG, London, United Kingdom, 21-25 Novembre 2009. Poster publié dans Gut. 58:a304.

Madi A, **Connil N** (2009). Implication de *Pseudomonas fluorescens* dans la pathogenèse de la maladie de Crohn. CECED, Strasbourg, 19-20 Mars 2009.

Guyard-Nicodème M, Veron W, Lesouhaitier O, Madi A, **Connil N**, Chapalain A, Tollemer H, Blier AS, Dagorn A, Leprettre C, Heitzmann A, Orange N, Feuilleley MGJ (2009). Toxicology of pyrogens and bacterial endotoxins: what do we measure? PDA Conference on Endotoxins, Paris, 17-18 Mars 2009.

Madi A, **Connil N**, Svinareff P, Orange N, Feuilleley MGJ (2008). Adhesion and cytotoxicity of *Pseudomonas fluorescens* on human intestinal Caco-2/TC7 cells. ESTIV INVITOX, 15th International Congress on In Vitro Toxicology, Stockholm, 25-28 Septembre 2008.

Connil N (2005). Régulation de la perméabilité intestinale par les bactéries probiotiques. Audition pour le prix jeune chercheur de l'Institut Français pour la Nutrition (IFN), Paris.

Connil N, Segain SP (2004). Régulation de la perméabilité paracellulaire intestinale par le cocktail probiotique VSL#3. Colloque du Club des Bactéries Lactiques, Nantes.

Connil N, Pilet MF, Brillet A, Leroi F, Dousset X, Prevost H (2004). Altération des produits de la mer par production microbienne d'amines biogènes. Congrès de la SFM (Société Française de Microbiologie), Bordeaux.

Connil N, Le Breton Y, Dousset X, Auffray Y, Rincé A, Prévost H (2002). First characterization from *Enterococcus faecalis* of a tyrosine decarboxylase operon in Pr 7th symposium on Lactic Acid Bacteria genetics, metabolism and applications, Amsterdam, Netherlands.

Connil N, Prévost H, Dousset X (2001). Bioprotection du saumon par *Carnobacterium divergens* V41 et suivi d'implantation par PCR-multiplex. Colloque AGORAL, Outils biologiques, procédés et techniques d'analyse, Dijon.

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7) TRAVAUX DE RECHERCHE

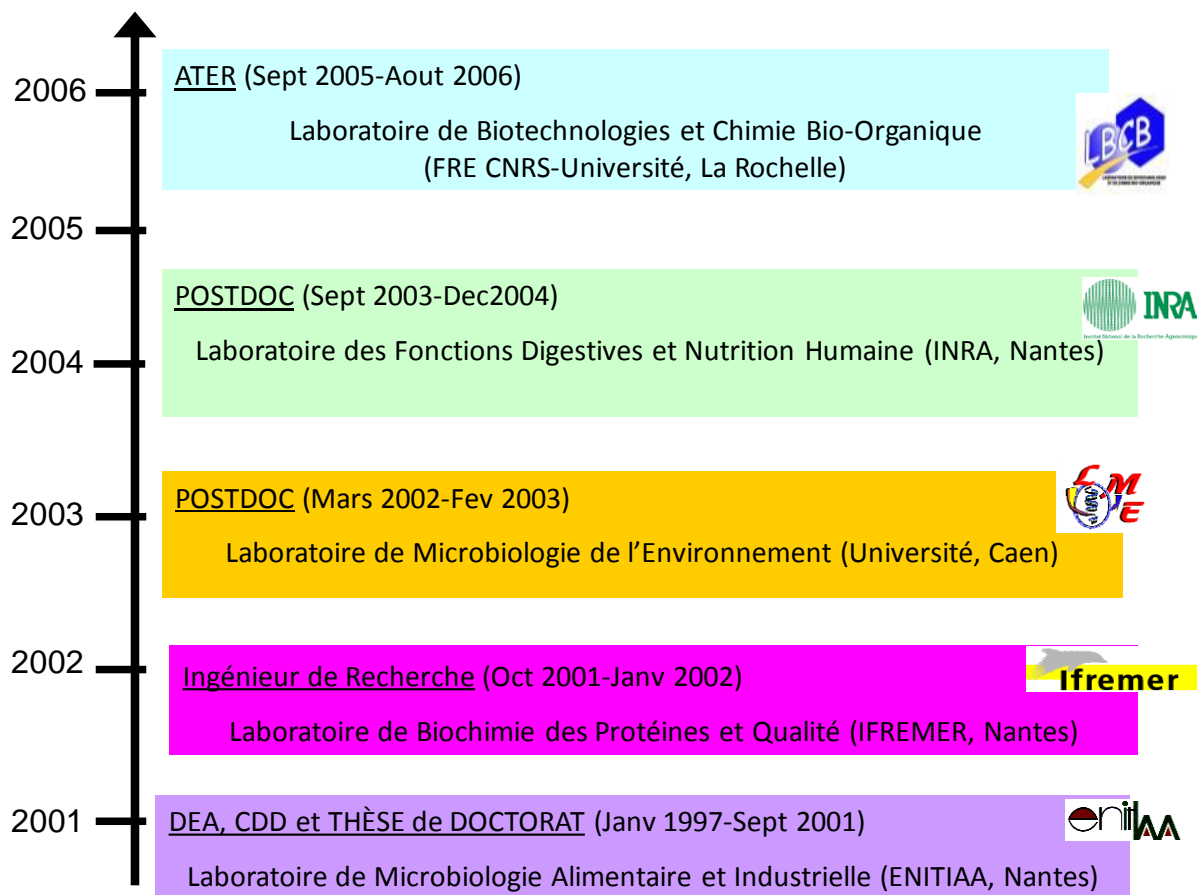


Figure 4 : Parcours scientifique antérieur à mon recrutement au LMSM

7-1) ACTIVITES DE RECHERCHE ANTERIEURES A MON RECRUTEMENT AU LMSM

Avant d'exposer ma recherche actuelle, je vais présenter brièvement mon parcours scientifique et les activités de recherche que j'ai réalisées avant mon recrutement au LMSM. J'ai commencé mes études universitaires en 1991 dans une petite faculté de Vendée où il n'existait pas de laboratoire de recherche et je pensais à l'époque m'orienter vers le DESS de contrôle qualité des aliments de l'université de Dijon. En 1996, je suis donc allée à Dijon pour faire une maîtrise de Biologie Cellulaire, Sciences et Techniques du Végétal et me préparer au concours d'entrée du DESS. Pendant cette année de formation, j'ai effectué un stage au Laboratoire de Génie des Procédés Biotechnologiques (LGPB) de l'ENSBANA sur l'étude de la perméabilité chez *Saccharomyces cerevisiae*, sous la direction du Dr Pierre-André Maréchal. Ceci m'a permis d'avoir un premier contact avec le monde de la recherche.

La même année, pendant les congés d'été, j'ai réalisé un second stage recherche au Laboratoire de Microbiologie des Sols (LMS) de l'INRA de Dijon où j'ai travaillé sur les interactions entre *Bradyrhizobium japonicum* et *Pseudomonas* (responsable scientifique : Dr Gérard Catroux). Ce stage m'a donné envie de me spécialiser en Microbiologie Fondamentale et Appliquée.

Je me suis donc inscrite au DEA de Microbiologie Fondamentale et Appliquée, commun aux universités de Caen, Rennes et Brest. Les enseignements ont eu lieu à Rennes et j'ai effectué mon stage au Laboratoire de Microbiologie Alimentaire et Industrielle (LMAI) de l'ENITIAA de Nantes où je suis restée par la suite en contrat à durée déterminée pendant quelques mois puis trois ans en thèse de doctorat.

Mon parcours scientifique depuis mon DEA et ma thèse de doctorat jusqu'à mon recrutement au LMSM est présenté sur la **Figure 4** et détaillé ci-dessous.

Activités de recherche effectuées au LMAI de l'ENITIAA (Janv 1997-Sept 2001)

Thème de recherche du DEA et CDD : Couplage hybridation in situ-cytométrie de flux pour une détection rapide et spécifique de *Carnobacterium divergens*, bactérie du saumon fumé.

Responsable scientifique : Dr Xavier Dousset

A mon arrivée au laboratoire de Microbiologie Alimentaire et Industrielle de l'ENITIAA, les techniques de biologie moléculaire y étaient peu développées et l'hybridation *in situ* n'était pas pratiquée. J'ai donc participé au développement de cet outil au sein du laboratoire après avoir suivi une semaine de formation à l'INRA de Theix chez le Dr Marie-Christine Montel et j'ai dû faire preuve d'autonomie expérimentale. Ce travail a abouti à la construction d'une sonde Cb spécifique du genre *Carnobacterium*, marquée à la fluorescéine et ciblant l'ARNr 16S, qui permettait de détecter et quantifier par hybridation *in situ*-cytométrie de flux *Carnobacterium divergens* V41 ou *Carnobacterium piscicola* V1 en cultures pures ou en mélange avec *Listeria innocua* F (Connil et al, 1998).

Thème de recherche de mon doctorat : Etude et caractérisation génétique préliminaire de la production de tyramine chez *Carnobacterium divergens* V41, souche au potentiel bioprotecteur dans le saumon fumé.

Responsables scientifiques : Pr Hervé Prévost et Dr Xavier Dousset

Carnobacterium divergens V41, bactérie isolée de viscères de poissons produit une bactériocine présentant une forte activité anti-*Listeria* : la divercine V41. L'utilisation de cette souche bactérienne pour la biopréservation du saumon fumé a donc été envisagée. Cependant, les bactéries du genre *Carnobacterium* sont productrices de tyramine, amine biogène nocive pour le consommateur si elle est ingérée en grande quantité.

Dans ce contexte, l'objectif scientifique de mon travail de thèse a été d'étudier la capacité de *C. divergens* V41 à s'implanter dans le saumon, d'évaluer sa capacité bioprotectrice (production de divercine V41) et son potentiel d'altération (production de tyramine). J'ai tout d'abord participé au développement d'une méthode moléculaire de PCR sur colonies, par amplification de régions intergéniques 16S-23S (ITS), pour identifier et quantifier rapidement les bactéries du genre *Carnobacterium* parmi la flore lactique. Par la suite, en couplant l'amplification des ITS à celle d'une région spécifique à la souche V41, située sur le

gène codant pour la divercine V41, j'ai pu par PCR-multiplex mettre au point la détection spécifique de la souche V41 parmi d'autres souches de *Carnobacterium*. Cette méthode m'a permis de montrer que la souche *C. divergens* V41 est compétitive vis-à-vis de la flore naturelle du saumon (Connil et al, 2002a).

Mes travaux ont montré par ailleurs que la souche *C. divergens* V41, inoculée dans des extraits de saumon fumé produit de la divercine V41 (bactériocine anti-*Listeria*) quelles que soient les conditions technologiques étudiées (NaCl, pH) et ne modifie ni quantitativement ni qualitativement les amines biogènes présentes dans le saumon fumé (Connil et al, 2002b). Cependant, la production potentielle de tyramine par *C. divergens* V41 pourrait constituer un obstacle à son utilisation pour la biopréservation du saumon fumé c'est pourquoi j'ai poursuivi mes travaux dans le sens d'une étude plus approfondie du métabolisme de la tyramine chez *C. divergens* V41. Afin de caractériser les déterminants génétiques impliqués dans le métabolisme de la tyramine chez *C. divergens* V41, des essais de mutagenèse génétique ont été envisagés. Cependant, *Carnobacterium* s'est révélé être une bactérie difficilement transformable pour espérer caractériser rapidement les déterminants génétiques recherchés. J'ai donc continué cette étude chez une bactérie phylogénétiquement proche de *Carnobacterium* et productrice de tyramine : *Enterococcus faecalis* JH2-2 grâce à une collaboration avec le Dr Alain Rincé du Laboratoire de Microbiologie de l'Environnement de l'université de Caen. Le criblage d'une banque de mutants intégratifs d'*E. faecalis* JH2-2 nous a permis d'identifier un ORF de 1860 pb contenant un gène que nous avons nommé *tdc* et qui semble coder pour une tyrosine décarboxylase potentielle. Ce résultat a fait l'objet d'une publication dans *Applied and Environmental Microbiology* (Connil et al, 2002c). C'est le premier gène codant pour une tyrosine décarboxylase qui a pu être caractérisé chez un procaryote.

Ces résultats ont permis d'initier la construction de sondes nucléiques pour identifier le gène *tdc* homologue chez *C. divergens* V41 et d'autres microorganismes producteurs de tyramine, et cribler plus aisément les flores productrices de tyramine dans les produits alimentaires.

Activité de recherche effectuée à l'IFREMER (Oct 2001-Janv 2002)

Thème de recherche : Caractérisation d'allergènes dans le surimi de cabillaud

Responsable scientifique : Dr Joël Fleurence

A la fin de ma thèse de doctorat, entre le dépôt de mon manuscrit et ma soutenance, j'ai eu l'opportunité de pouvoir effectuer un remplacement de congé maternité (contrat à durée déterminée de 3 mois) au Laboratoire de Biochimie des Protéines et Qualité (LBPQ) à l'IFREMER de Nantes sur un poste de cadre de recherche. Ce travail m'a permis de me former à des techniques biochimiques (chromatographies, western blot et dot blot). J'ai participé aux premières étapes de purification des protéines allergènes du surimi de cabillaud par gel filtration sur colonne Sephadex 200 et chromatographie échangeuse d'anions. Le poisson est l'un des aliments les plus incriminés dans les allergies alimentaires et le laboratoire cherchait à purifier et caractériser des allergènes encore inconnus chez le cabillaud.

Activité de recherche effectuée à l'Université de Caen (Mars 2002-Fev 2003)

Thème de recherche : Réponse au stress oxydatif chez *Lactobacillus casei* BL23

Responsable scientifique : Pr Axel Hartke

Afin d'approfondir mes compétences en Biologie Moléculaire, j'ai effectué par la suite un contrat postdoctoral d'un an, au Laboratoire de Microbiologie de l'Environnement (LME), laboratoire avec lequel j'avais eu des contacts et collaborations durant ma thèse. La viabilité des microorganismes probiotiques dans les produits commercialisés est souvent faible en raison de l'accumulation d'acide lactique, de l'abaissement du pH, et du stress oxydatif généré par la perméabilité de l'emballage à l'oxygène et la production de peroxyde d'hydrogène (H_2O_2) par la flore lactique. Malgré l'utilisation massive de *L. casei* dans l'industrie alimentaire, la réponse de cette bactérie envers un stress acide ou oxydatif n'était pas bien connue. J'ai donc démarré une étude sur le potentiel de survie de *L. casei* BL23 envers les stress acide (acide lactique) et oxydatif (H_2O_2), celle-ci a montré une résistance accrue de la bactérie en phase stationnaire. Une adaptation en phase exponentielle à une dose subléthale en H_2O_2 permet également d'accroître la résistance de la bactérie à un stress H_2O_2 ultérieur.

Par la suite, j'ai exprimé chez *L. casei* BL23, le gène *sodA* de *Lactococcus lactis* MG1363 qui code pour une superoxyde dismutase manganèse dépendante (MnSod) en utilisant le vecteur d'expression pMSp3535 inductible par la nisine qui n'avait encore jamais été testé chez *L. casei*. La superoxyde dismutase, enzyme naturellement absente chez *L. casei* BL23, constitue un mécanisme physiologique clé de défense cellulaire contre le stress oxydatif. Ces travaux ont conduit à une expression effective nisine dépendante du gène *sodA* chez *L. casei* BL23 conférant ainsi à la bactérie une résistance accrue contre un stress oxydatif.

Activité de recherche postdoctorale à l'INRA de Nantes (Sept 2003-Dec 2004)

Thème de recherche : Régulation de la perméabilité intestinale par les probiotiques

Responsable scientifique : Dr Jean-Pierre Segain

A la fin de mon contrat postdoctoral au LME, j'ai pris contact avec le Laboratoire des Fonctions Digestives et Nutrition Humaine (LFDNH) de l'INRA de Nantes, pour proposer mes compétences en Microbiologie et Biologie Moléculaire et me former en retour à la Biologie Cellulaire des Eucaryotes. En collaboration avec le Dr Jean-Pierre Segain, chargé de recherche dans ce laboratoire, j'ai monté un projet qui m'a permis d'obtenir le prix Jeune Chercheur 2003 de l'Institut Français de Nutrition pour financer mon travail de postdoctorant pendant un an. Mon étude a eu pour objectif de mieux comprendre comment les bactéries probiotiques modulent la barrière intestinale et pourraient ainsi exercer des effets bénéfiques dans certaines pathologies digestives. J'ai développé au laboratoire une méthode *in vitro* de culture sur filtres de cellules intestinales différenciées (HT29-clone19A) permettant d'étudier leur perméabilité par mesure de résistance transépithéliale et de flux paracellulaire. Après différenciation, les cellules ont été traitées en apical par des bactéries probiotiques (cocktail VSL#3 et *Lactobacillus casei* BL23) ou les surnageants de ces bactéries. Dans les deux cas, les résultats ont montré une forte réduction de la perméabilité paracellulaire (augmentation de la résistance transépithéliale) des cellules HT29-clone19A laissant suggérer l'implication d'un facteur soluble sécrété par les probiotiques, et pouvant intervenir dans le dialogue probiotique-cellule épithéliale. L'analyse transcriptionnelle des constituants des jonctions serrées impliqués dans la perméabilité paracellulaire : ZO-1, occludine et claudine-1 a été effectuée par RT-PCR quantitative, et a montré qu'après 7 h de traitement probiotique, leur expression est significativement augmentée.

Après fixation des cellules au paraformaldéhyde 3,7%, le cytosquelette d'actine marqué à la Phalloïdine-Alexa 488 a été observé au microscope à fluorescence et nous avons pu montrer que le traitement probiotique renforce le réseau d'actine. Ce type d'analyses constitue une base rationnelle pour aider à la sélection et l'utilisation de nouveaux probiotiques en nutrition préventive ou thérapeutique.

Activité de recherche à l'Université de La Rochelle (Sept 2005-Aout 2006)

Thème de recherche : Etude du mode d'action de la microcine L

Responsable scientifique : Dr Sophie Sablé

Mon contrat postdoctoral à l'INRA de Nantes étant seulement financé pour une durée d'un an, j'ai postulé par la suite sur des contrats d'ATER et j'ai été recruté comme ½ ATER au Laboratoire de Biotechnologies et Chimie Bio-organique (LBCB) de l'Université de La Rochelle. Le sujet de recherche qui m'a été confié était l'étude du mode d'action de la microcine L. Cette bactériocine a été isolée et purifiée à partir de la souche *Escherichia coli* LR05 qui produit également trois autres bactériocines : les microcines B17, J25 et D93. La microcine L est active contre *Salmonella enterica*, des souches de *Shigella* et *Pseudomonas* et *Escherichia coli* enteropathogènes. Etant donné son spectre d'action, la microcine L semblait intéressante pour une utilisation future en médecine humaine et/ou vétérinaire. Avant d'envisager son utilisation, il était important de mieux comprendre son mode d'action. De nombreuses bactériocines agissent sur la perméabilité et le potentiel membranaire, mais certaines peuvent avoir une action intracellulaire au niveau de la réplication de l'ADN, de la synthèse protéique ou de la division cellulaire. Dans ce contexte, mon travail effectué en collaboration avec une doctorante du laboratoire a consisté à produire et purifier de grandes quantités de peptides (5-10 mg) par chromatographie liquide haute pression, qui devaient être utilisés par la suite pour fabriquer des anticorps contre la microcine L, afin d'aller détecter la cible de ce peptide antimicrobien sur des souches sensibles à la bactériocine.

Suite à mon doctorat, j'ai donc eu une succession de quatre « activités postdoctorales » sur la période d'octobre 2001 à août 2006. Des contrats de quelques mois ou un an sont très courts pour démarrer une étude bibliographique sur un nouveau sujet, réaliser les travaux de recherche et aboutir à un projet de publication. Par contre, cette mobilité m'a permis de pouvoir comparer la façon de fonctionner de différents laboratoires universitaires (université de Caen et université de La Rochelle) et organismes de recherche (IFREMER, INRA), apprendre de nombreuses techniques en Microbiologie, Biologie Moléculaire, Biologie Cellulaire et Biochimie, avoir une ouverture thématique dans la façon d'appréhender un sujet de recherche, et créer de nouveaux contacts humains et professionnels pour des collaborations futures. Ainsi, ce qui pouvait être ressenti à l'époque comme une faiblesse, s'est avéré à posteriori un avantage dans ma carrière de chercheur.

7-2) ACTIVITES DE RECHERCHE EFFECTUEES AU LMSM

Mon positionnement au sein du laboratoire

J'ai été recruté au LMSM (ex-LMDF), EA4312 de l'université de Rouen en 2006 pour y développer un projet sur *Pseudomonas* et l'intestin. Le LMSM est une mono-équipe dont la thématique générale de recherche actuelle concerne le rôle de la communication et des facteurs environnementaux ou eucaryotes dans l'adaptation et la virulence bactériennes. Ce laboratoire a fortement évolué au cours du contrat quadriennal 2008-2011, et du contrat quinquennal 2012-2016. Créé initialement en 1986 sur le site de l'IUT d'Evreux, sous le nom de « Laboratoire de Microbiologie du Froid » ou LMDF par les Pr. Jeannine Guespin et Nicole Orange, sa thématique principale de recherche portait sur les études physiologique, moléculaire et génétique de l'adaptation des bactéries psychrotrophes aux domaines thermiques.

A mon arrivée au LMDF (2006), le laboratoire était dirigé par le Pr Nicole Orange. En plus de salles dédiées à des techniques de microbiologie, biochimie et biologie moléculaire, le LMDF bénéficiait d'une pièce de culture cellulaire qui avait été installée quelques années auparavant par le Pr Marc Feuilloley et le Dr Olivier Lesouhaitier afin de pouvoir étudier les interactions bactéries-cellules eucaryotes. Cet axe du laboratoire avait été défini sous le terme de « Microbiologie Cellulaire ». C'est à ce groupe de travail que j'ai été associée lors de mon recrutement, afin d'y développer les interactions bactéries-intestin en complément aux études sur cellules gliales qui y étaient effectuées.

En 2008, la direction du laboratoire a été reprise par le Pr Marc Feuilloley et l'équipe s'est recentrée sur l'étude de l'adaptation des bactéries à leur microenvironnement, depuis la perception et transmission du stimulus, jusqu'à la régulation des gènes codant pour les protéines impliquées dans la réponse à ces signaux. Le laboratoire a alors pris pour identifiant LMDF-SME pour « Laboratoire de Microbiologie du Froid-Signaux et Microenvironnement » qui correspondait mieux à la thématique du laboratoire. En 2012, suite à l'évaluation AERES, le label du laboratoire a été reconduit avec la thématique générale de recherche suivante « étude du rôle de la communication et des facteurs environnementaux ou eucaryotes dans l'adaptation et la virulence bactériennes ».

Le laboratoire a alors pris le nom de LMSM pour « Laboratoire de Microbiologie Signaux et Microenvironnement » par souci de simplification, les initiales DF pour « du Froid » ne se justifiant plus étant donné que le laboratoire ne travaillait plus seulement sur des aspects d'adaptation au froid. Au 30 juin 2015, le LMSM regroupe 45 membres (Hors stagiaires) dont 10 PR ou MCF-HDR, 2 PR-émerites, 6 MCF non HDR, 9 IATOS permanents ou CDD et 3 membres associés professeurs agrégés (PRAG) (Figure 5).

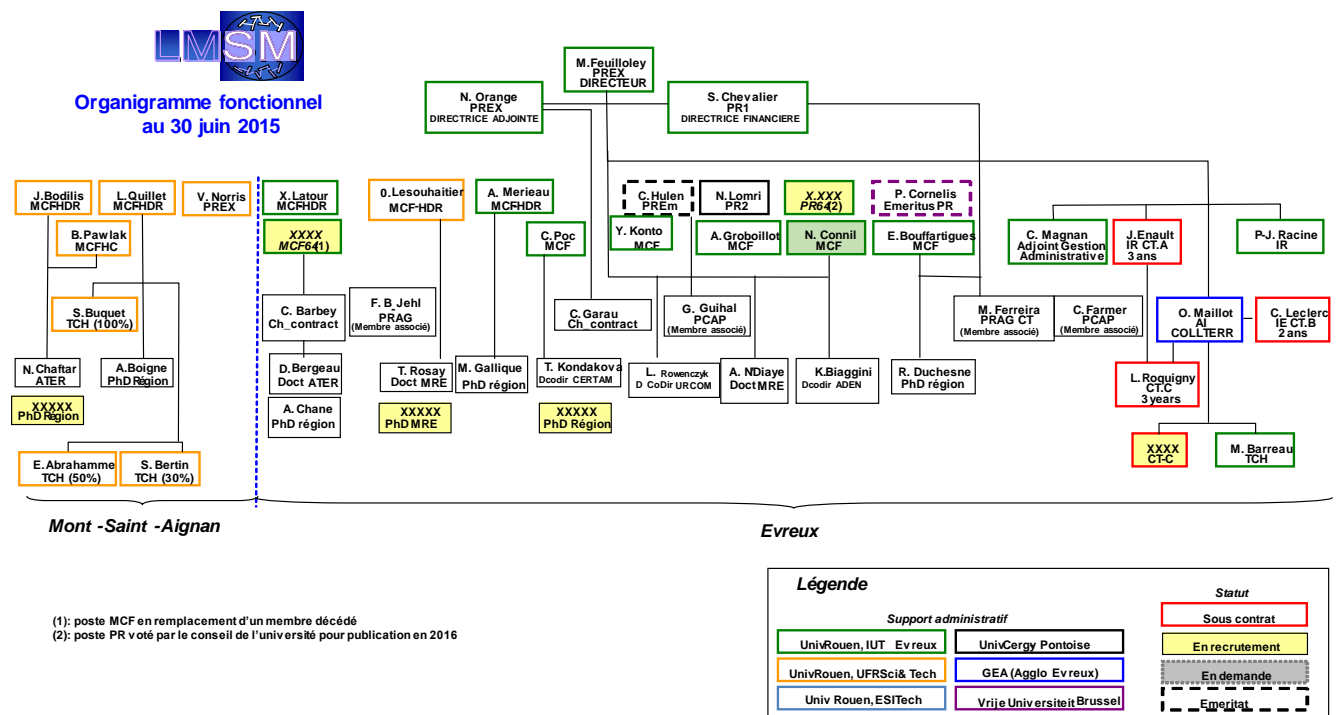


Figure 5 : Organigramme du LMSM (version HCERES 2015, modifiée)

A cet effectif, s'ajoute 3 à 6 post-doctorants, 10 doctorants, ainsi que chaque année, une moyenne de 6 étudiants stagiaires de niveau L3 à M2. A la fin de l'année 2015, ou au cours de l'année 2016, le laboratoire va emménager dans de nouveaux locaux au sein du « Centre de Sécurité Sanitaire (PRT27) ». Il sera alors possible de travailler non seulement sur des bactéries de niveau 2, mais aussi si nécessaire, sur des bactéries hautement pathogènes puisque le LMSM disposera d'un laboratoire de sécurité de niveau L3.

Actuellement, les travaux de recherche effectués au sein du LMSM sont centrés sur les systèmes senseurs membranaires et leurs mécanismes de transduction, et sur les mécanismes de réponse bactériens impliqués dans l'expression de la virulence.

Ces processus sont explorés dans 4 modèles complémentaires d'interaction bactérie-hôte (peau, intestin, poumon et rhizosphère), et pourront peut-être permettre de mieux comprendre, comment une bactérie peut évoluer d'un état commensal ou avirulent vers un état pathogène selon son microenvironnement. Le modèle bactérien principalement étudié au LMSM est le genre *Pseudomonas* qui est présent dans ses 4 environnements.

Au sein du LMSM, je suis responsable du volet recherche axé sur *Pseudomonas* et l'intestin.

Le contexte scientifique de mes travaux : Etat de l'art sur *Pseudomonas* et l'intestin

Le genre *Pseudomonas* appartient au phylum des *Proteobacteria*, classe des γ -*Proteobacteria*, ordre des *Pseudomonadales*, famille des *Pseudomonadaceae*. Ce sont des bacilles à Gram négatif, non sporulés, mobiles grâce à un ou plusieurs flagelles polaires. Grâce à une grande richesse en voies métaboliques et capacité d'adaptation, les bactéries du genre *Pseudomonas* notamment *P. aeruginosa*, *P. fluorescens* et *P. putida* sont capables de coloniser des niches écologiques très diverses comme les environnements aquatiques, le sol, l'air, mais elles peuvent aussi être associées à des organismes eucaryotes (végétaux, animaux, insectes) (Silby et al, 2011) **(Figure 6)**.

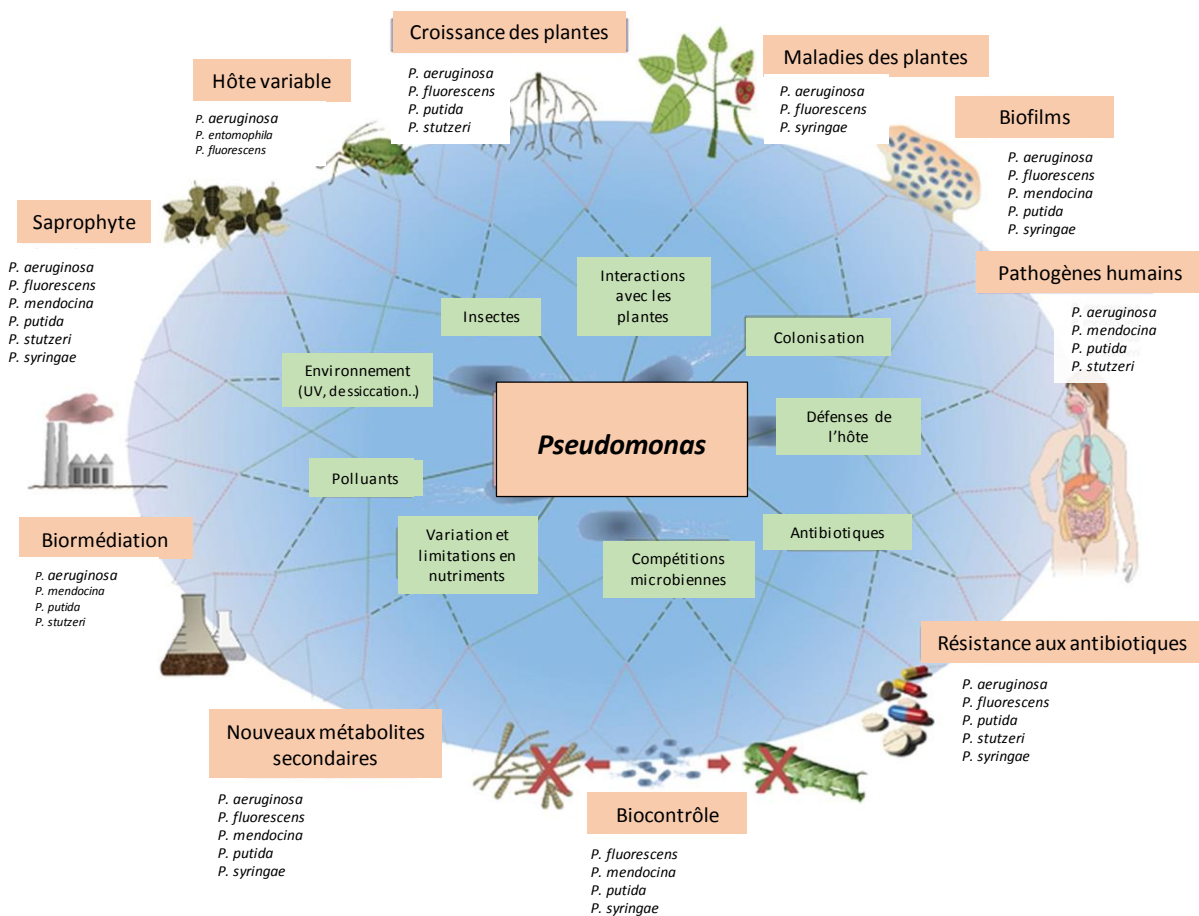


Figure 6 : Diversité des niches écologiques et fonctions chez les bactéries du genre *Pseudomonas* (Silby et al, 2011, modifié)

Le genre *Pseudomonas* est très hétérogène et contient plus d'une centaine d'espèces différentes, malgré de nombreux remaniements phylogénétiques, qui se poursuivent encore à l'heure actuelle grâce au séquençage et à l'analyse du génome total (Anzai et al, 2000, Silby et al, 2011). Des travaux récents de phylogénie, basés sur l'analyse des ARNr16S auxquels ont été soustraites les régions hypervariables montrent que le genre *Pseudomonas* peut être subdivisé en trois clusters distincts (Bodilis et al, 2012) (**Figure 7**).

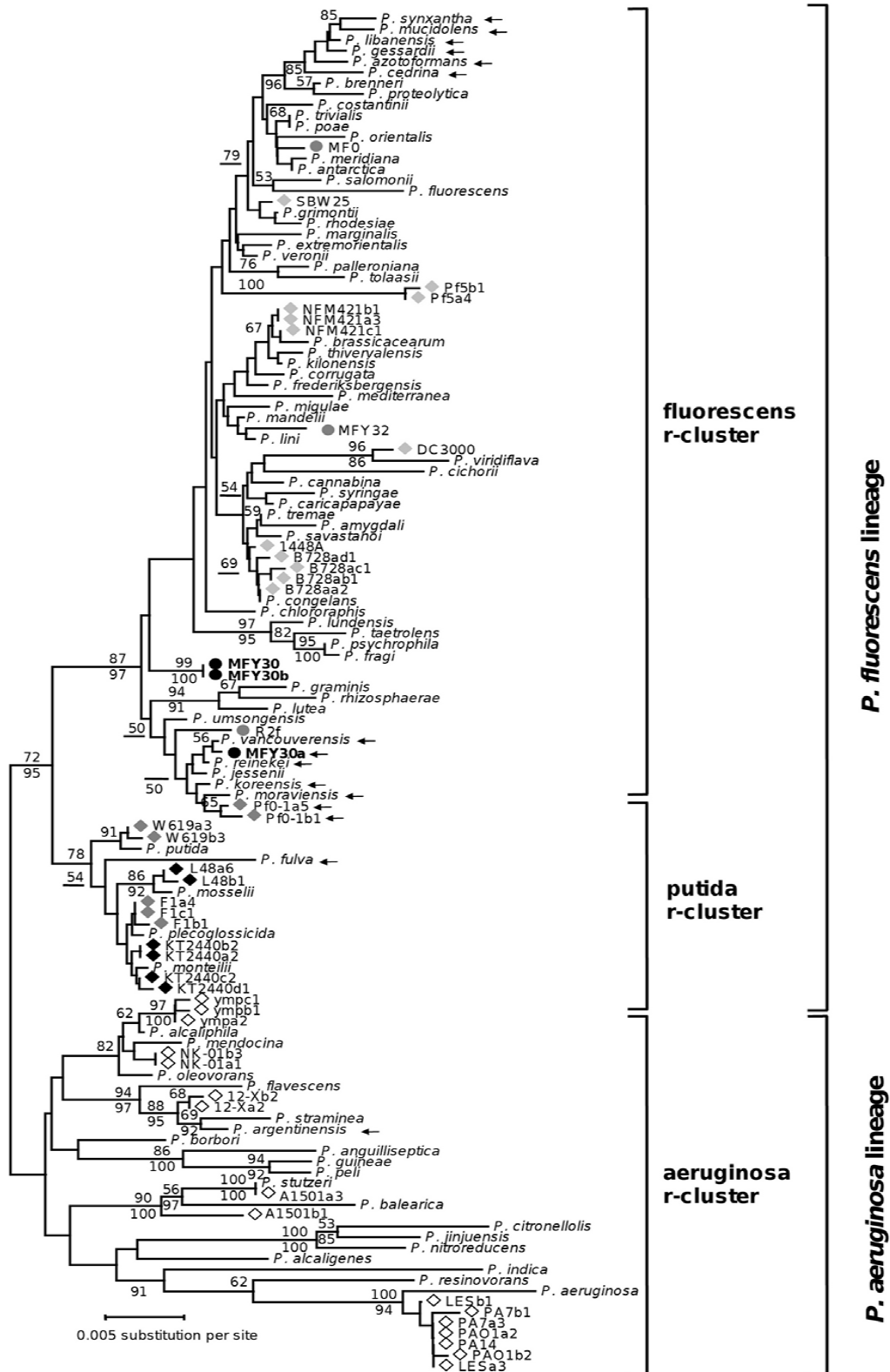


Figure 7 : Phylogénie des bactéries du genre *Pseudomonas* (Bodilis et al, 2012)

Ces 3 clusters sont :

- le cluster *fluorescens* dans lequel on retrouve plusieurs espèces dont *P. fluorescens*
- le cluster *putida* dans lequel est classé *P. mosselii* et quelques autres espèces
- et le cluster *P. aeruginosa* qui comprend entre autres l'espèce *P. aeruginosa*.

Le cluster *putida* est relié au cluster *fluorescens* pour former un grand groupe, « la lignée *fluorescens* ».

* *Pseudomonas aeruginosa* est l'espèce « type » du genre *Pseudomonas*. C'est l'espèce la plus connue et la mieux caractérisée. Elle est facilement identifiable par des méthodes phénotypiques et génotypiques. C'est un germe retrouvé essentiellement dans l'eau et le sol, et qui peut infecter un grand nombre d'organismes, parmi lesquels, l'homme, les souris, les nématodes et les plantes. Chez l'homme, *P. aeruginosa* (ou bacille pyocyanique) est un pathogène opportuniste capable d'infecter une grande variété de tissus et de sites différents (Lyczak et al, 2000). C'est un microorganisme mésophile dont la température optimale de croissance est de 37°C et qui peut croître jusqu'à 41°C, il est donc bien adapté à la température physiologique humaine. Cette bactérie est pratiquement inoffensive chez un individu sain mais se révèle redoutable chez les sujets immuno-déprimés ou ceux souffrant d'infections chroniques comme la mucoviscidose (Emerson et al, 2002). En France, *P. aeruginosa* occupe la troisième place dans la survenue d'infections nosocomiales après *Escherichia coli* et *Staphylococcus aureus* (InVS, Enquête nationale de prévalence des infections nosocomiales et des traitements anti-infectieux en établissements de santé, France, mai-juin 2012). Au niveau de l'intestin, *P. aeruginosa* est présent parmi les bactéries transitoires mais peut aussi être associé à la muqueuse intestinale (Gao et al, 2015).

Dans ce contexte, j'ai donc choisi d'inclure cette bactérie dans certains des travaux que j'ai encadrés au LMSM. En tant que pathogène opportuniste humain le mieux caractérisé parmi le genre *Pseudomonas* elle m'a permis d'avoir une base de comparaison pour l'étude d'autres espèces du genre *Pseudomonas* telles que *P. fluorescens* et *P. mosselii*.

* *Pseudomonas fluorescens* est une espèce décrite comme psychrotrophe, pouvant se développer à 4°C, avec une température optimale de croissance comprise entre 25 et 32°C. Cependant, certaines souches sont capables de survivre, voire se multiplier à 37°C. L'espèce *P. fluorescens* est très hétérogène, et a été subdivisée en cinq biovars sur la base de leurs phénotypes et activités biochimiques. La grande diversité génétique au sein de l'espèce *P. fluorescens* a suggéré de redéfinir la barrière de cette espèce, mais jusqu'à présent les analyses effectuées se sont avérées insuffisantes pour reclasser *P. fluorescens* en plusieurs autres espèces (Silby et al, 2011). Majoritairement retrouvées au sein de la rhizosphère, certaines souches de *P. fluorescens* ont un rôle phytoprotecteur, par la biosynthèse d'antifongiques elles limitent la prolifération des moisissures (Walsh et al, 2001). Cependant, *P. fluorescens* appartient aussi à la flore d'altération de certains aliments comme le fromage (Martin et al, 2011), les températures de conservation des denrées convenant à sa nature psychrotrophe. Quelques souches ont été décrites comme pathogènes des oiseaux et des poissons (Sun et Sun, 2015). Longtemps considérées comme non pathogènes chez l'homme, certaines souches de *P. fluorescens* ont été associées à des accidents transfusionnels (Puckett et al, 1992), et plus récemment à des infections pulmonaires (Bahrani-Mougeot et al, 2007) et cardiaques (Benito et al, 2012).

En 2000, une séquence d'origine microbienne nommée I2, spécifique de *P. fluorescens* a été identifiée dans une lésion de la muqueuse du colon d'un patient atteint de la maladie de Crohn (Sutton et al, 2000). Dans cette même étude, I2 était présent à la fois dans des régions lésées et saines de l'iléon, ce qui laisse supposer que *P. fluorescens* peut être présent en tant que commensal de l'intestin, et associé à la muqueuse. Un rôle de *P. fluorescens* dans la pathogénèse de la maladie de Crohn a été suggéré (Wei et al, 2002) et des travaux complémentaires ont montré que des anticorps anti-I2 étaient présents dans le sérum de 54% des patients atteints de la maladie de Crohn et pouvaient être associés au diagnostic sur la progression et la sévérité de la maladie (Arnott et al, 2004). Toutefois, à la suite de ces travaux, aucune autre étude n'a été entreprise sur l'implication éventuelle de *P. fluorescens* dans la maladie de Crohn. Il est en effet très complexe de pouvoir identifier précisément ce qui déclenche cette pathologie intestinale multifactorielle. Des prédispositions génétiques ont été mises en évidence, dont la mutation du gène CARD15/NOD2 qui est impliqué dans l'immunité innée, et qui code pour une protéine senseur intra-cytoplasmique pouvant reconnaître des bactéries de la lumière intestinale via une interaction avec leur LPS

(lipopolysaccharide). La maladie de Crohn est liée à la fois à des facteurs environnementaux (tabac, stress, alimentation) et à une réponse immunitaire inappropriée envers des bactéries de la flore commensale de l'intestin (Ananthakrishnan, 2015). Un facteur infectieux a également été suspecté, de nombreuses bactéries ont été mises en cause, et plus récemment une dysbiose, c'est-à-dire des modifications qualitatives et quantitatives de la composition du microbiote intestinal (Seksik et al, 2006). Dans ce contexte, à mon arrivée au laboratoire (2006), j'ai souhaité étudier les interactions de *P. fluorescens* avec l'intestin et évaluer son potentiel pathogène. Des souches de *P. fluorescens* d'origine intestinale n'étant pas disponibles dans la littérature et/ou les collections internationales, j'ai sélectionné pour cette étude, deux souches de *P. fluorescens* présentes au laboratoire, la souche psychrotrophe *P. fluorescens* MF37 ayant été isolé du lait (Burini et al, 1994) et la souche *P. fluorescens* MFN1032, provenant du CHU de Rouen, et issue d'expectorations purulentes (Chapalain et al, 2008), et ceci dans le but de pouvoir comparer leur comportement vis-à-vis de cellules intestinales.

* *P. mosselii* est une espèce qui s'apparente phylogénétiquement à *P. fluorescens* (Dabboussi et al, 2002) ou *P. putida* (Bodilis et al, 2012) selon les auteurs. Elle est capable de croître dans une gamme de température de 4 à 41°C avec une température optimale de croissance de 30°C. Isolée pour la première fois en 2002 à partir d'échantillons cliniques (Dabboussi et al, 2002), elle a aussi été identifiée comme agent causal d'une endocardite suite à la pose d'une prothèse cardiaque en 2009 (McLellan et Partridge, 2009). Multi-résistante aux β -lactamines, elle a été suspectée comme vecteur potentiel de transfert de gènes de résistance vers des bactéries pathogènes (Giani et al, 2012). Certaines souches sont pathogènes du poisson *Oreochromis niloticus* (Soto-Rodriguez et al, 2013), ou encore du nématode entomopathogène, *Rhabditis* sp. (Tambong, 2013). Sa virulence chez l'homme n'ayant jamais été étudiée, j'ai trouvé intéressant d'ajouter cette bactérie à certains de mes travaux de recherche.

Suite à cette introduction permettant de préciser le contexte scientifique au sein du laboratoire, et l'état de l'art concernant la thématique *Pseudomonas* et l'intestin, voici une présentation des différents projets de recherche que j'ai menés au sein du LMSM.

(Par souci de clarté, dans la suite de ce document, j'ai fait apparaître en bleu, les thèses que j'ai encadrées, les collaborations de recherche en lien avec ces projets et les articles scientifiques qui en ont découlés).

A) Adhésion et cytotoxicité de *Pseudomonas fluorescens* sur l'intestin, potentiel inflammatoire et voies de signalisation associées (Article 1, Madi et al, 2010a), Figure 8

L'adhésion sur les cellules épithéliales intestinales étant une étape cruciale dans les processus infectieux, nous avons étudié le potentiel d'adhésion de *P. fluorescens* sur les lignées intestinales Caco-2/TC7 et HT-29. Dans le cadre de la thèse d'Amar Madi, que j'ai encadré avec le Pr Marc Feuilloley, nous avons pu montrer que la souche environnementale psychrotrophe *P. fluorescens* MF37 et la souche clinique *P. fluorescens* MFN1032 sont capables d'adhérer de façon similaire aux cellules Caco-2/TC7. Au contraire sur le modèle cellulaire HT29 qui sécrète plus de mucus, l'adhésion était plus élevée pour *P. fluorescens* MFN1032 que pour *P. fluorescens* MF37. La cytotoxicité sur ces cellules a également été étudiée, et a montré un pouvoir cytotoxique dose-dépendante et souche-dépendante, avec une mortalité cellulaire maximale de 30% après 24 h d'infection des cellules Caco-2/TC7 avec *P. fluorescens* MF37 et une mortalité cellulaire maximale de 70% dans le cas de la souche clinique *P. fluorescens* MFN1032. Ces résultats nous ont permis de conclure que *P. fluorescens* MFN1032 a un comportement qui se rapproche de *P. aeruginosa* PAO1, un pathogène opportuniste bien caractérisé. Les deux souches de *P. fluorescens* étudiés étaient capables d'induire la production d'interleukine-8 (IL-8) chez les cellules Caco-2/TC7 et HT-29 montrant donc aussi un potentiel pro-inflammatoire non négligeable.

L'expression du gène IL-8 est régulée principalement par les facteurs de transcription NF- κ B et AP-1 (dépendant de la voie des MAPK). En collaboration avec le Dr Hervé Blottière de l'INRA de Jouy en Josas, nous avons donc recherché la voie de signalisation qui est activée par ces bactéries et nous avons pu montrer que dans nos conditions expérimentales celles-ci activaient la voie de signalisation AP-1 des MAPK et n'activaient pas la voie NF- κ B (Figure 8) contrairement à *P. aeruginosa* PAO1. Les MAPK sont connues pour être impliquée notamment dans l'infection de certaines bactéries invasives comme *Salmonella enterica* serovar *typhimurium* ou *Listeria monocytogenes* dans les cellules épithéliales intestinales (Hobbie et al, 1997 ; Tang et al, 1998) ou dans les macrophages (Schwan et al, 1996).

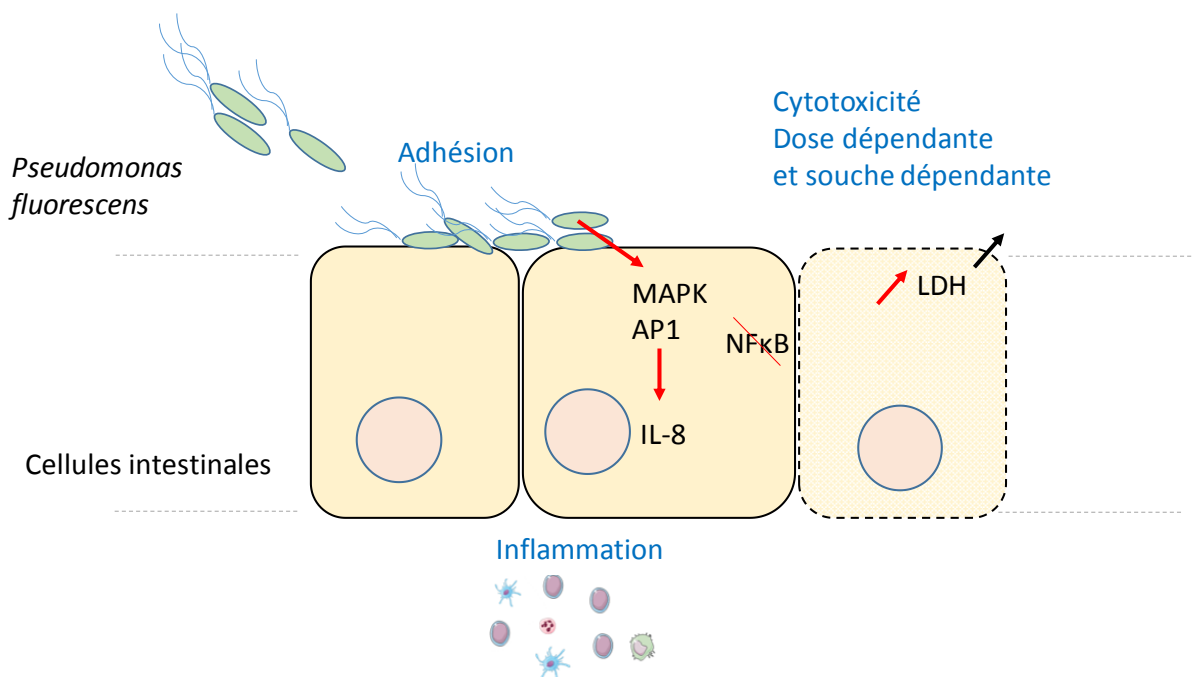


Figure 8 : Adhésion et cytotoxicité de *Pseudomonas fluorescens* sur les cellules de l'intestin, potentiel inflammatoire et voies de signalisation associées (schéma simplifié)

(Article 1, Madi et al, 2010a)

Madi A, Lakhdari O, Blottière HM, Guyard-Nicodème M, Le Roux K, Groboillot A, Svinareff P, Doré J, Orange N, Feuilloley MG, Connil N (2010). The clinical *Pseudomonas fluorescens* MFN1032 strain exerts a cytotoxic effect on epithelial intestinal cells and induces Interleukin-8 via the AP-1 signaling pathway. BMC Microbiol. 10:215.

B) Invasion et translocation de *Pseudomonas fluorescens*. Effet sur la résistance transépithéliale (TER) et le cytosquelette d'actine F (Article 2, Madi et al, 2010b) Figure 9

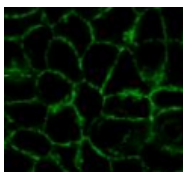
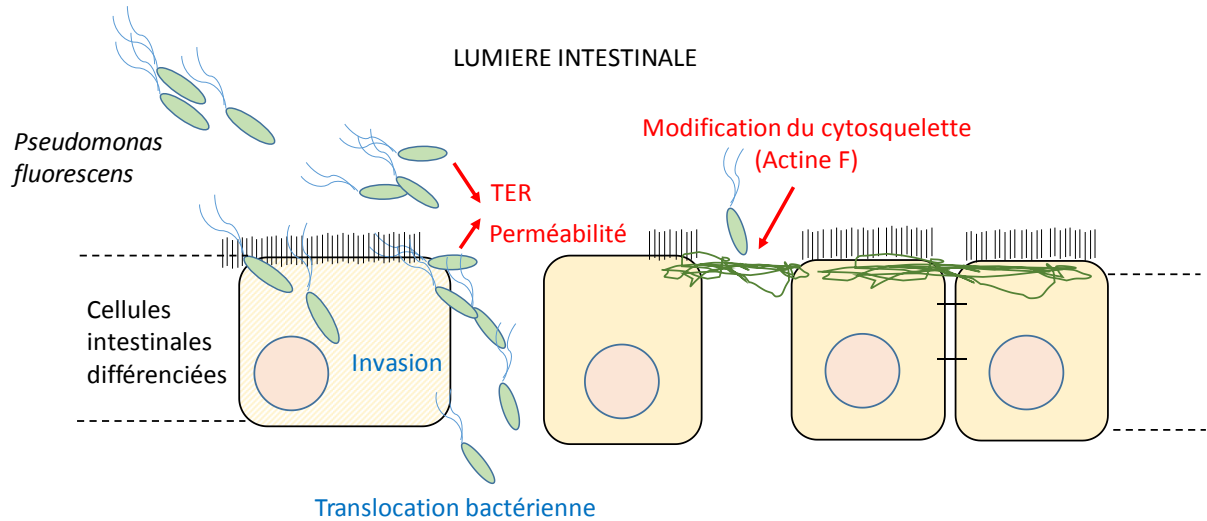
Toujours dans le cadre de la thèse d'Amar Madi, nous avons étudié l'effet de *P. fluorescens* sur des cellules Caco-2/TC7 cultivées sur inserts de culture pendant 21 jours, un modèle de cellules différenciées qui se rapproche plus des entérocytes de l'intestin que celui utilisé précédemment. Nous avons pu ainsi montrer que *P. fluorescens* diminue la résistance transépithéliale (TER) de la monocouche de cellules Caco-2/TC7 d'environ 20% après 24 h d'infection. La diminution de la TER était associée à une augmentation de la perméabilité des cellules Caco-2/TC7 (Figure 9), celle-ci a été mesurée par le passage de billes de FITC-dextran 4 kDa du compartiment apical vers le compartiment basolatéral.

Le marquage de l'actine F avec de la phalloïdine couplée à l'Alexa-488 a permis d'observer une réorganisation du cytosquelette. Le cytosquelette d'actine F est intimement lié aux protéines des jonctions serrées qui participent dans la régulation de la perméabilité paracellulaire, Nous avons donc recherché si l'expression des gènes ZO-1, occludine et claudine-1, qui codent pour certaines protéines des jonctions serrées, est modifiée suite à l'infection par *P. fluorescens*. Les résultats ont montré que l'expression des gènes ZO-1 et occludine augmente de façon non significative 4 h après l'infection avec *P. fluorescens* mais que l'expression de claudine-1 était diminuée. L'infection de cellules Caco-2 avec *E. coli* entero-invasive conduit également à une diminution de la claudine-1 et à une baisse de la TER (Qin et al, 2009).

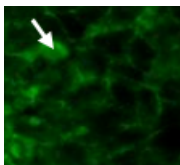
La capacité de translocation de *P. fluorescens* a été évaluée après 6 h d'infection et a montré que ces bactéries sont capables de traverser la couche de cellules Caco-2/TC7 (Figure 9).

Ceci pourrait permettre d'expliquer au moins en partie comment l'antigène I2 spécifique de *P. fluorescens* est retrouvé dans le sérum de certains patients atteints de la maladie de Crohn. Notre étude a montré aussi que *P. fluorescens* présente un comportement invasif plus important vis-à-vis de cellules Caco-2/TC7 différenciées cultivées sur inserts que dans le cas de cellules confluentes indifférenciées cultivées sur plaques.

Alors qu'une lyse cellulaire importante avait pu être observée, lorsque des cellules cultivées sur plaques étaient infectées par *P. fluorescens*, aucune cytotoxicité n'a été détectée de façon significative lors de l'infection des cellules différenciées par *P. fluorescens*.



Cytosquelette d'actine F des cellules non infectées



Réorganisation du cytosquelette d'actine F des cellules infectées avec *P. fluorescens* MFN1032

Figure 9 : Invasion et translocation de *Pseudomonas fluorescens* dans l'intestin. Effet sur la résistance transépithéliale et le cytosquelette d'actine F (schéma simplifié)

(Article 2, Madi et al, 2010b)

Madi A, Svinareff P, Orange N, Feuilloley MG, Connil N (2010). *Pseudomonas fluorescens* alters epithelial permeability and translocates across Caco-2/TC7 intestinal cells. Gut Pathog. 2(1):16.

Au contraire, *P. aeruginosa* entraîne une lyse cellulaire sur les cellules différenciées, et même si celle-ci reste plus modérée que dans le cas de cellules cultivées sur plaques, elle pourrait permettre d'expliquer la baisse plus forte de la TER avec cette bactérie. Les cellules intestinales peuvent avoir une réponse variable à l'infection en fonction de leur niveau de différenciation. En effet, il a été montré précédemment, que la différenciation et la polarisation de la barrière épithéliale augmentent la résistance de ces cellules vis-à-vis d'une infection par *Pseudomonas* (Kazmierczak et al, 2004).

C) *Lactobacillus salivarius* SMXD51, un probiotique potentiel qui peut réduire les effets délétères de *Pseudomonas* sur l'épithélium intestinal (Article 3, Messaoudi et al, 2012)

Lactobacillus salivarius SMXD51 est une bactérie qui a été isolée à partir de caecum de poulets tunisiens (Messaoudi et al, 2011) et présente l'intérêt de produire une bactériocine, la salivaricine SMXD51, qui est active contre *Campylobacter jejuni*, *Listeria monocytogenes*, *Staphylococcus aureus* et *Salmonella*. Cette souche bactérienne pourrait donc être une bonne alternative à l'utilisation d'antibiotiques dans les élevages.

En collaboration avec les Pr Xavier Dousset et Hervé Prévost du laboratoire Secalim de Nantes, et le Pr Mohammed Manai de l'Université de Tunis, j'ai participé à la mise en évidence du potentiel probiotique de *L. salivarius* SMXD51 et à l'encadrement de Soumaya Messaoudi, une doctorante extérieure au LMSM.

Nous avons montré que cette bactérie pouvait tolérer les conditions gastro-intestinales (acidité et bile), était capable d'adhérer aux cellules intestinales et de stimuler le système immunitaire (induction de β -défensine-2). Un traitement des cellules Caco-2/TC7 différenciées avec *L. salivarius* SMXD51 a entraîné une augmentation de la résistance transépithéliale, et un renforcement du cytosquelette d'actine F (**Figure 10, B**) comparé aux cellules contrôles non traitées avec la bactérie (**Figure 10, A**). *L. salivarius* SMXD51 remplit les principales conditions, généralement requises pour un probiotique. De plus, lorsque *L. salivarius* SMXD51 a été ajoutée sur des cellules Caco-2/TC7 en prétraitement avant une infection par *Pseudomonas*, elle a permis de réduire l'effet délétère du pathogène sur l'épithélium (**Figure 10, C et D**) (Messaoudi et al, 2012). Dans une étude complémentaire, il serait intéressant de voir si le même effet peut être obtenu lors d'une infection avec *C. jejuni* ou un autre pathogène et de compléter ces résultats par des expériences *in vivo*.

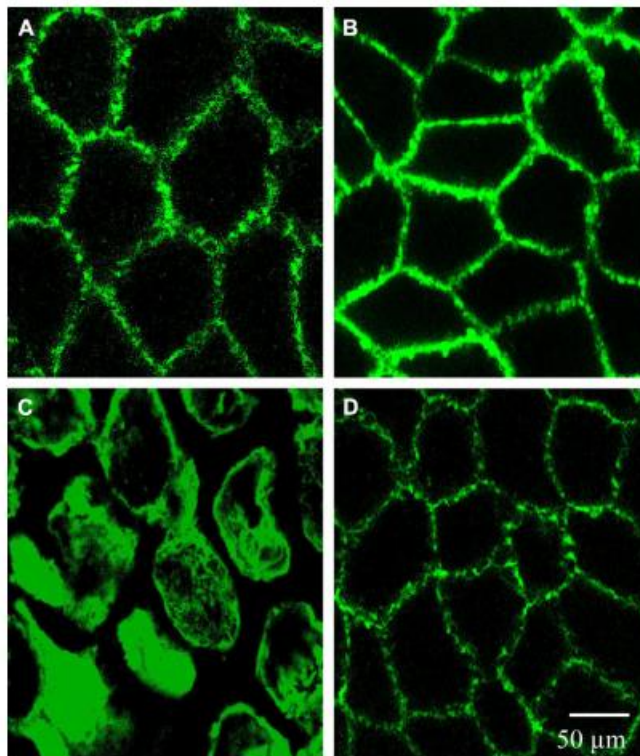


Figure 10 : Marquage du cytosquelette d'actine F avec la phalloidine Alexa 488, chez des cellules Caco-2/TC7, contrôles non traitées avec une bactérie (A), traitées avec *L. salivarius* SMXD51 (10⁸ CFU/ml) (B), infectées avec *P. aeruginosa* PAO1 (10⁶ CFU/ml) (C), et prétraitées avec SMXD51 avant infection par PAO1.

(Article 3, Messaoudi et al, 2012)

Messaoudi S, Madi A, Prévost H, Feuilloley M, Manai M, Dousset X, Connil N (2012). *In vitro* evaluation of the probiotic potential of *Lactobacillus salivarius* SMXD51. *Anaerobe*. 18(6):584-589.

D) *Pseudomonas mosselii*, un pathogène émergent ?

(Article 4, Leneveu-Jenvrin et al, 2013)

P. mosselii est une nouvelle espèce du genre *Pseudomonas* qui a été caractérisée en 2002 (Dabboussi et al, 2002). Des analyses de l'ADNr16S, oprF et oprD ont montré que *P. mosselii* a un lien phylogénétique avec des souches cliniques de *P. putida*. Un cas d'endocardite due à la pose d'une valve cardiaque a été associé à *P. mosselii* en 2009 (McLellan and Partridge, 2009), suite à cela il a été suggéré que cette bactérie pourrait être un pathogène potentiel. A peu près à la même période, une étude effectuée sur des biopsies de l'iléon d'enfants atteints de la maladie de Crohn a permis d'identifier la présence de séquence d'ADNr16S de plusieurs bactéries du genre *Pseudomonas* dont une proche de celle de *P. mosselii* (Wagner et al, 2008). Au sein du LMSM, il avait été montré que certaines souches de *P. mosselii* sont capables d'adhérer et entraîner une nécrose sur des cellules gliales de rat (Chapalain et al, 2008). Pour compléter ces travaux, nous avons étudié, **dans le cadre de la thèse de Charlène Leneveu-Jenvrin que j'ai encadrée avec le Pr Sylvie Laurency**, le comportement de deux souches de *P. mosselii* sur les cellules Caco-2/TC7 : *P. mosselii* ATCC BAA-99, une souche clinique ayant été isolée chez un patient souffrant d'infections pulmonaires (Dabboussi et al, 2002) ; et *P. mosselii* MFY161 collectée à l'hôpital Charles Nicolle de Rouen, à partir de l'urine d'un patient souffrant d'une hépatite alcoolique. Les résultats ont montré que les deux souches de *P. mosselii* étudiées présentaient une activité cytotoxique sur les cellules Caco-2/TC7. La lyse cellulaire la plus forte a été obtenue avec *P. mosselii* MFY161 (environ 65% de cytotoxicité). Cette valeur reste bien en dessous de celle de *P. aeruginosa* PAO1 qui entraîne une lyse de 86% dans ces mêmes conditions, mais est très similaire aux résultats trouvés précédemment pour la souche clinique *P. fluorescens* MFN1032. *P. mosselii* ATCC BAA-99 et *P. mosselii* MFY161 se sont montrées peu invasives, et n'ont pas entraîné la production des cytokines IL-6 et IL-8. Par contre, ces deux bactéries ont provoqué une altération de la perméabilité épithéliale des cellules Caco-2/TC7 différenciées et se sont révélées être résistantes à de nombreux antibiotiques (**Figure 11**). Ceci est cohérent avec l'étude de Giani et al (Giani et al, 2012) qui avait suggéré que *P. mosselii* et certains pathogènes opportunistes inhabituels pourraient agir comme vecteur de transfert de gènes de résistance aux beta-lactamines.

Antibiotic Strain (μg)	TIC 75	PRL 75	CT 50	IPM 10	ATM 30	TOB 10	GN 15	AK 30	TIM 85	CAZ 30	CIP 5	CFS 30	LEV 5	SXT 25	FF 50	NET 30
ATCC BAA-99	R	I	S	S	S	S	S	S	R	S	S	R	S	R	R	R
MFY161	R	I	R	S	S	S	S	S	R	S	S	R	S	R	R	I

Figure 11 : Résistance aux antibiotiques chez *P. mosselii* ATCC BAA-99 et *P. mosselii* MFY161. Antibiotiques testés : ticarcilline (TIC), piperacilline (PRL), colistine (CT), imipenème (IPM), aztreoname (ATM), tobramycine (TOB), gentamycine (GN), amikacine (AK), ticarcilline+acide clavulanique (TIM), ceftazidime (CAZ), ciprofloxacine (CIP), cefsulodine (CFS), levofloxacine (LEV), trimethoprim-sulphamethoxazole (SXT), fosfomycine (FF) et netilmicine (NET).

R: Résistant, I: Intermediaire, S: sensible (classement selon les recommandations de l'antibiogramme de la Société Française de Microbiologie, CA-SFM, 2012).

Ainsi, ces travaux sur *P. mosselii* et les précédents effectués sur *P. fluorescens* montrent qu'il ne faut pas négliger le suivi en milieu hospitalier de ces deux espèces bactériennes, même si leur pouvoir pathogène reste généralement limité comparé à celui de *P. aeruginosa*.

E) Etude *in-vivo* et *ex-vivo*. Effet de *Pseudomonas fluorescens* sur la perméabilité intestinale. Implication de NOD2. (Article 5, Alnabhani et al, 2015)

En collaboration avec le Pr Jean-Pierre Hugot, le Dr Frederick Barreau, et le Dr Ziad Alnabhani de l'unité INSERM UMR1149, nous avons effectué des expériences *in-vivo* et *ex-vivo* chez la souris (**Figure 12**), pour essayer de mieux comprendre les interactions possibles entre *P. fluorescens* et l'intestin, et pour voir si les effets délétères observés précédemment sur modèles *in vitro* pouvaient avoir une réelle signification biologique dans un organisme en cas d'infection. Les résultats obtenus ont montré qu'une infection par *P. fluorescens* MFN1032 est capable d'augmenter la perméabilité paracellulaire au niveau de l'iléon et des plaques de Peyer des souris C57BL/6. Les mécanismes en cause ont été recherchés et nous avons pu observer une augmentation de la sécrétion de TNF- α et d'IL-1 β , ainsi que de l'expression de la MLCK (myosin light chain kinase) qui est connue pour son rôle dans la régulation de la perméabilité paracellulaire en réponse à l'IL-1 β et aux pathogènes (Meinzer et al, 2012 ; Jung et al, 2012). Enfin, cette étude a montré que l'expression d'IL-1 β par les macrophages infectés par *P. fluorescens* MFN1032 est dépendante de l'expression de *Nod2* dans le compartiment hématopoïétique. Ainsi *P. fluorescens* MFN1032 semble altérer l'homéostasie de la barrière épithéliale par un mécanisme similaire à celui observé précédemment chez *Yersinia pseudotuberculosis* (Meinzer et al, 2012). Ces auteurs ont montré en effet que l'acétylation de RICK (un effecteur situé en aval de *Nod2*) par le facteur de virulence YopJ de *Yersinia* réduit son affinité pour NOD2. Quand RICK est inhibé, NOD2 est capable de former un complexe avec la caspase-1, ce qui conduit à la production d'IL-1 β mature. Au vu de ces résultats, on peut supposer que des aliments réfrigérés contenant des bactéries telles que *Pseudomonas* ou *Yersinia* pourraient entraîner une augmentation de la perméabilité et de l'inflammation intestinales chez des personnes sensibles. Ceci conforte l'hypothèse émise précédemment d'un rôle potentiel des bactéries psychrotrophes et de la chaîne du froid dans la maladie de Crohn (Hugot et al, 2003).

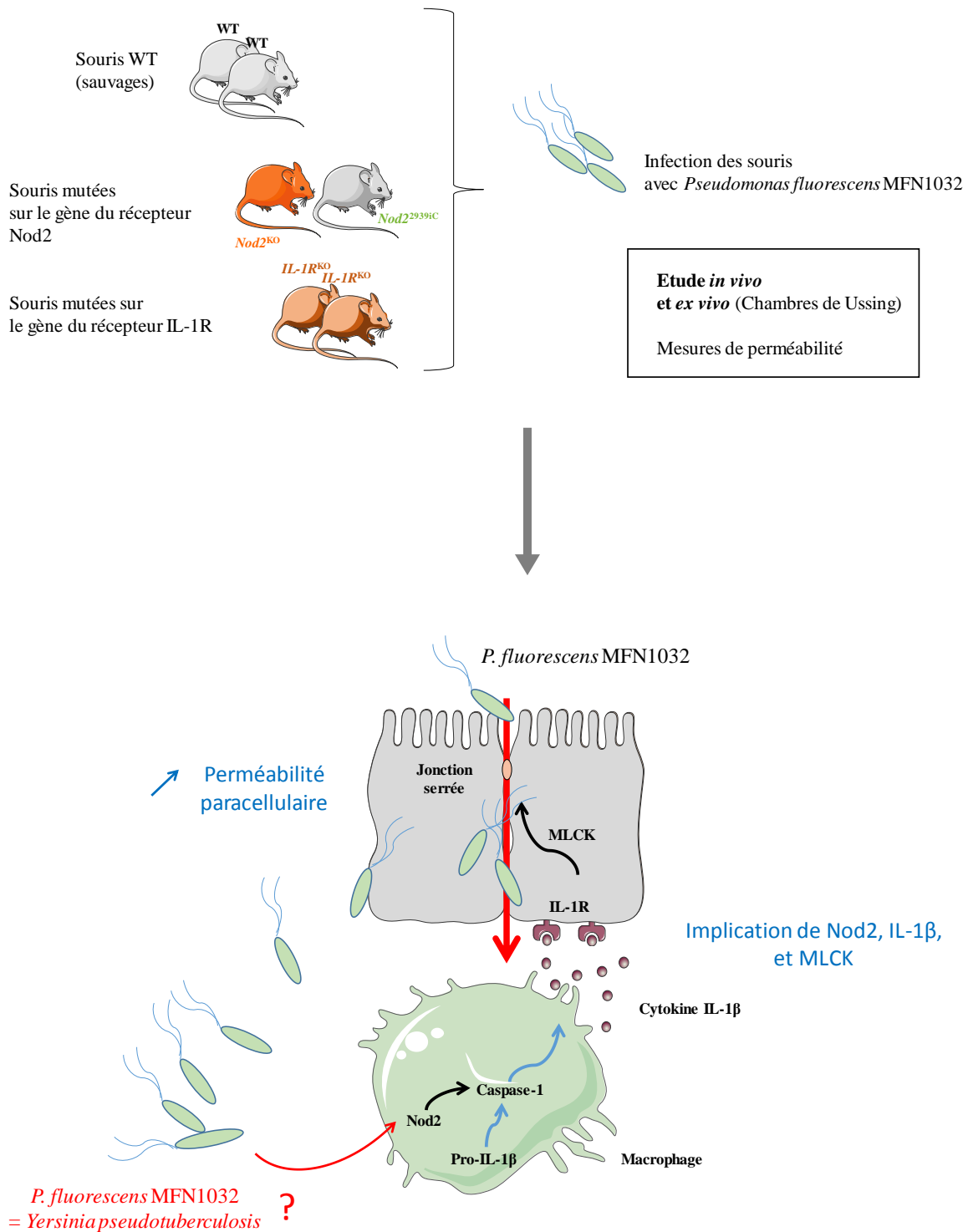


Figure 12 : Etude *in-vivo* et *ex-vivo*. Effet de *Pseudomonas fluorescens* sur la perméabilité intestinale via Nod2, IL-1 β et MLCK (schéma simplifié) (Article 5, Alnabhani et al, 2015)

Alnabhani Z, Montcuquet N, Biaggini K, Dussailant M, Roy M, Ogier-Denis E, Madi A, Jallane A, Feuilloley M, Hugot JP, Connil N, Barreau F (2015). *Pseudomonas fluorescens* alters the intestinal barrier function by modulating IL-1 β expression through hematopoietic NOD2 signaling. *Inflamm Bowel Dis.* 21(3):543-555

F) Effet des facteurs intestinaux sur *Pseudomonas*

Les espèces du genre *Pseudomonas* peuvent s'adapter à des environnements très divers grâce à leur très grande plasticité génétique et à l'arsenal de facteurs de virulence dont elles disposent. Dans les années 1990, certains chercheurs ont commencé à s'intéresser à l'effet de molécules eucaryotes sur la virulence bactérienne (Lyte et Ernst, 1992 ; Freestone et Lyte, 2008), et ceci a donné naissance à un nouveau champ disciplinaire : l'endocrinologie microbienne. Au sein du laboratoire, ce travail a été initié en 2002, par le Dr Olivier Lesouhaitier et le Pr Marc Feuilloley, pour étudier l'effet de neuropeptides et neuromodulateurs sur *Pseudomonas*. Ils ont ainsi pu montrer que les peptides natriurétiques BNP et CNP (Veron et al, 2007 ; Veron et al, 2008 ; Lesouhaitier et al, 2009) et l'acide γ -aminobutyrique GABA (Dagorn et al, 2013) sont capables de moduler la virulence de *P. fluorescens* et/ou *P. aeruginosa*.

Aucune étude interne ou externe au laboratoire n'ayant concerné l'effet des facteurs intestinaux sur *Pseudomonas*, j'ai décidé de développer cet axe à partir de 2008, pour être en accord avec la politique du LMSM, et son recentrage thématique sur l'effet du microenvironnement sur l'adaptation et la virulence bactériennes.

-Effet de la β -défensine-2 humaine sur *P. fluorescens* (Article 6, Madi et al, 2013)

Parmi la diversité de molécules et de peptides présents dans l'intestin, les défensines constituent une famille de peptides antimicrobiens naturels largement impliqués dans l'immunité innée. Chez l'homme, ces peptides qui possèdent un large spectre d'activité antimicrobienne, sont connus pour jouer un rôle dans le contrôle de la diversité du microbiote intestinal et peuvent être produits par les cellules épithéliales en contact avec la flore intestinale résidente ou de passage. Dans ce contexte, au cours de la [thèse d'Amar Madi et du master 2 de Ziad Alnabhani](#), nous avons cherché à déterminer l'effet de la β -défensine-2 humaine (hBD-2) sur la croissance et la virulence de *Pseudomonas*. Les résultats ont montré que la croissance de *P. fluorescens* n'était pas inhibée en présence de hBD-2 pour des concentrations inférieures à 100 $\mu\text{g}/\text{mL}$. De façon similaire, De Smet et al (2005) avaient également observé que *P. aeruginosa* n'était sensible aux défensines qu'à partir d'une concentration de 250 $\mu\text{g}/\text{mL}$. Dans notre étude, nous avons pu montrer que l'infection des cellules Caco-2/TC7 par *P. fluorescens* MF37 et *P. fluorescens* MFN1032 entraînait une

production de hBD-2 par ces cellules, via l'activation de AP-1 et de la voie des MAPK. Parallèlement à cela, de façon inattendue, nous avons pu observer que si on traite *P. fluorescens* MF37 par hBD-2 avant l'infection des cellules Caco-2/TC7, le pouvoir cytotoxique et inflammatoire de cette bactérie est augmenté, ce qui n'est pas le cas avec la souche clinique *P. fluorescens* MFN1032. Nous avons donc émis l'hypothèse que contrairement à la souche environnementale *P. fluorescens* MF37, la souche clinique *P. fluorescens* MFN1032 pourrait être déjà adaptée à l'environnement humain et à la sécrétion des défensines humaines, et sa virulence pourrait ainsi être déjà fortement exprimée suite à cette adaptation. Cette proposition semble cohérente avec le fait que lors d'une infection des cellules Caco-2/TC7 en situation basale (c'est-à-dire par des bactéries non traitées par hBD-2), nous avons trouvé précédemment que la cytotoxicité de la souche MFN1032 était beaucoup plus élevée que celle de la souche MF37. Ces travaux montrent que certaines souches de *P. fluorescens* pourraient être capables de détourner à leur avantage la production de hBD-2. De manière intéressante, dans une autre étude, il avait été montré aussi que *P. aeruginosa* est capable d'échapper à la réponse immunitaire de l'hôte en manipulant l'expression de hBD-2 (Dössel et al, 2012).

-Effet de la sérotonine, la substance P et l'épinéphrine sur *P. fluorescens*

(Article 7, Biaggini et al, 2015)

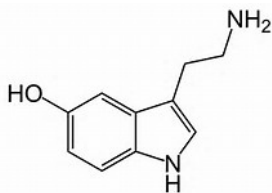
Dans le cadre de la thèse de Kelly Biaggini que je dirige actuellement avec le Pr Pierre Déchelotte, nous avons sélectionné un certain nombre de molécules produites par le système entéro-endocrinien afin d'étudier leur effet sur *P. fluorescens* et parmi celles-ci la sérotonine (5-hydroxytryptamine), la substance P, et l'épinéphrine/adrénaline (ou β ,3,4-trihydroxy-*N*-méthylphenethylamine) (**Figure 13**).

* La sérotonine joue le rôle de neurotransmetteur dans le système nerveux central. Cependant on la retrouve aussi au niveau de la muqueuse gastro-intestinale où elle représente 80% de la sérotonine totale de l'organisme, et sa production serait influencée par le microbiote intestinal (O'Mahony et al, 2015).

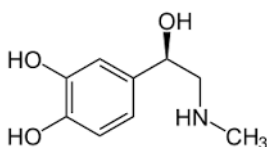
* La substance P est un neuropeptide associé à de nombreuses fonctions physiologiques parmi lesquelles la régulation de l'anxiété et de la douleur. Connue pour avoir un rôle dans la communication bidirectionnelle intestin-cerveau, la substance P pourrait agir sur le microbiote intestinal (Holzer et Farzi, 2014). Une étude publiée en 2008 a montré que la

substance P ainsi que d'autres neuropeptides comme le neuropeptide Y, le peptide vasoactif intestinal (VIP), la neurokinine A peuvent avoir une action antibactérienne contre de nombreux microorganismes de la peau, de l'intestin et des poumons (El Karim et al, 2008). La substance P est également présente dans la sueur, et des travaux récents réalisés au LMSM ont montré que ce peptide augmente fortement la virulence de *Bacillus cereus*, *Staphylococcus aureus* et *Staphylococcus epidermidis* sur des kératinocytes en culture (Mijouin et al, 2013).

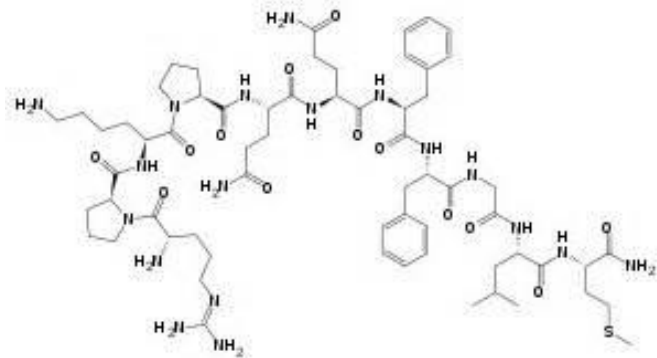
* L'épinéphrine (adrénaline) fait partie de la famille des Catécholamines qui comprend aussi notamment la norépinéphrine (noradrénaline) et la dopamine. Chez *Escherichia coli* O157:H7, l'épinéphrine et la norépinéphrine augmentent la mobilité bactérienne, la formation de biofilms et sa virulence (Bansal et al, 2007). Un senseur à ces molécules (nommé QseC) a été identifié chez cette bactérie et une analyse bioinformatique a montré des homologues à ce senseur présent chez plus de 25 souches pathogènes de l'homme ou des plantes (Rasko et al, 2008 ; Curtis et al, 2014) et aussi chez *P. fluorescens* F113, une souche biocontrôle de la rhizosphère (**Figure 14**).



Sérotonine
5-hydroxytryptamine
Formule chimique: C₁₀H₁₂N₂O
Masse molaire: 176,22 g/mol



Épinéphrine/adrénaline
β,3,4-trihydroxy-N-méthylphenethylamine
Formule chimique: C₉H₁₃NO₃
Masse molaire: 183,20 g/mol



Substance P
Formule chimique: C₆₃H₉₈N₁₈O₁₃
Masse molaire: 1 348 g/mol

Figure 13 : Structures chimiques de la sérotonine, la substance P et l'épinéphrine/adrénaline

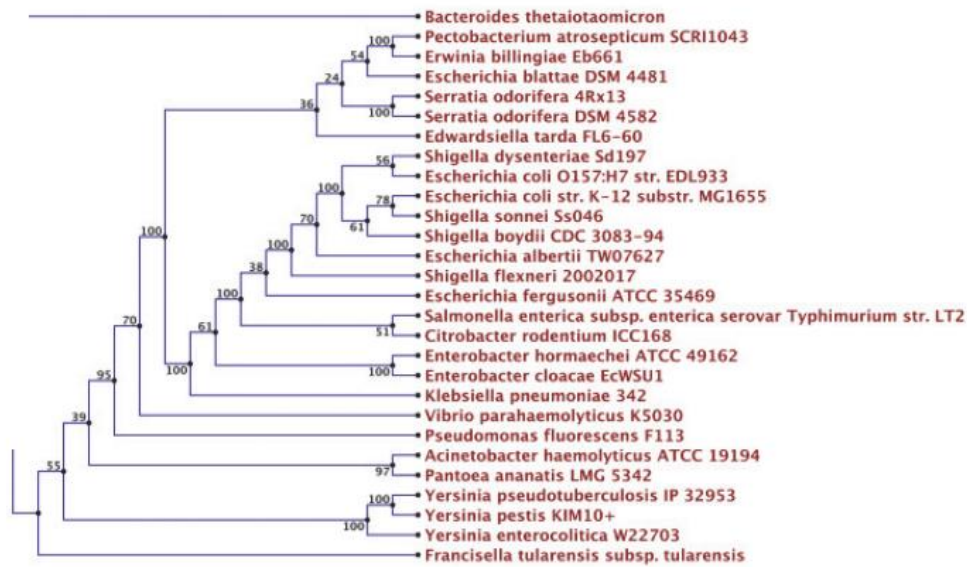


Figure 14 : Bactéries présentant des homologues de Qsec

(Des homologues de Qsec sont retrouvés chez des pathogènes de l'homme ou des plantes mais aussi chez des souches biocontrôles de la rhizosphère comme *P. fluorescens* F113).

Les résultats de nos travaux sur l'effet de la sérotonine, la substance P et l'épinéphrine/adrénaline sur *P. fluorescens*, ont montré que la croissance de la bactérie n'est pas modifiée en présence de sérotonine, substance P ou épinéphrine/adrénaline utilisés à des doses physiologiques. Par contre, ces molécules modulent sa mobilité de type « swim » et/ou « swarm ». La substance P augmente l'activité proinflammatoire de *P. fluorescens* MFN1032 sur des cellules Caco-2/TC7 (sécrétion d'IL-8), alors que les autres molécules testées (sérotonine et épinéphrine/adrénaline) n'ont aucun effet sur ce paramètre.

Comme nous l'avons décrit précédemment ([Article 2, Madi et al, 2010b](#)), lorsque les cellules Caco-2/TC7 sont infectées avec *P. fluorescens* MFN1032 pendant 24 h, ceci entraîne une baisse de la résistance transépithéliale/une augmentation de la perméabilité de la monocouche cellulaire. Cet effet est potentialisé lorsque la bactérie est prétraitée avec la substance P ou l'épinéphrine et une dégradation importante de la monocouche de cellules et du cytosquelette d'actine-F a pu être observée.

G) La protéine translocatrice (TSPO) chez *Pseudomonas fluorescens*

(Article 8, Leneveu-Jenvrin et al, 2014 ; Article 9, Leneveu-Jenvrin et al, 2015)

P. fluorescens possède dans son génome le gène *tspo*, codant pour une protéine homologue de la protéine translocatrice TSPO mitochondriale. Une analyse bioinformatique réalisée au sein de notre laboratoire a mis en évidence la présence de ce gène dans 97 génomes séquencés eubactériens (Chapalain et al, 2009). On le retrouve chez des bactéries très diverses (**Figure 15**), comme *Rhodobacter sphaeroides* et *Sinorhizobium meliloti* chez les α -protéobactéries, *Anabaena variabilis* chez les cyanobactéries, ou bien encore *Enterococcus faecalis*, *Staphylococcus haemolyticus*, *Bacillus subtilis*, *Lactobacillus plantarum* chez les firmicutes. Le gène *tspo* est présent chez certaines γ -protéobactéries, dont *P. fluorescens* et *P. syringae* mais semble absent chez *P. aeruginosa* et *P. mosselii*.

Chez les Eucaryotes, le gène *tspo* code pour la protéine TSPO, impliquée dans le transport du cholestérol et des porphyrines, la mort cellulaire, le stress et l'adaptation. Chez *Rhodobacter sphaeroides*, la protéine TSPO est principalement produite en condition d'anoxie et semble agir comme un régulateur négatif de la biosynthèse des pigments photosynthétiques en réponse à une diminution de la tension en oxygène (Yeliseev et Kaplan, 1995). Elle régule également le flux de porphyrines chez cette bactérie, les pigments photosynthétiques étant formés à partir de précurseurs porphyriques. Chez *Sinorhizobium meliloti*, TSPO contrôle avec FixL un locus nommé *ndi* pour « nutrient deprivation induced locus » dont l'expression est induite en réponse à des stress environnementaux, comme la carence en nutriments, la diminution de la tension en oxygène ou encore un stress osmotique (Davey et de Bruijn, 2000). La fonction de TSPO chez les firmicutes n'a pas été étudiée pour le moment et les principaux travaux concernant la protéine TSPO chez les γ -protéobactéries ont été effectués sur *P. fluorescens* au sein de notre laboratoire (Leneveu-Jenvrin et al, 2014). Chez cette bactérie, la protéine TSPO présente une certaine conservation structurale et fonctionnelle avec la TSPO mitochondriale. Elle est impliquée dans l'adhésion bactérienne et semble être liée au potentiel cytotoxique de la bactérie sur les cellules gliales (Chapalain et al, 2009).

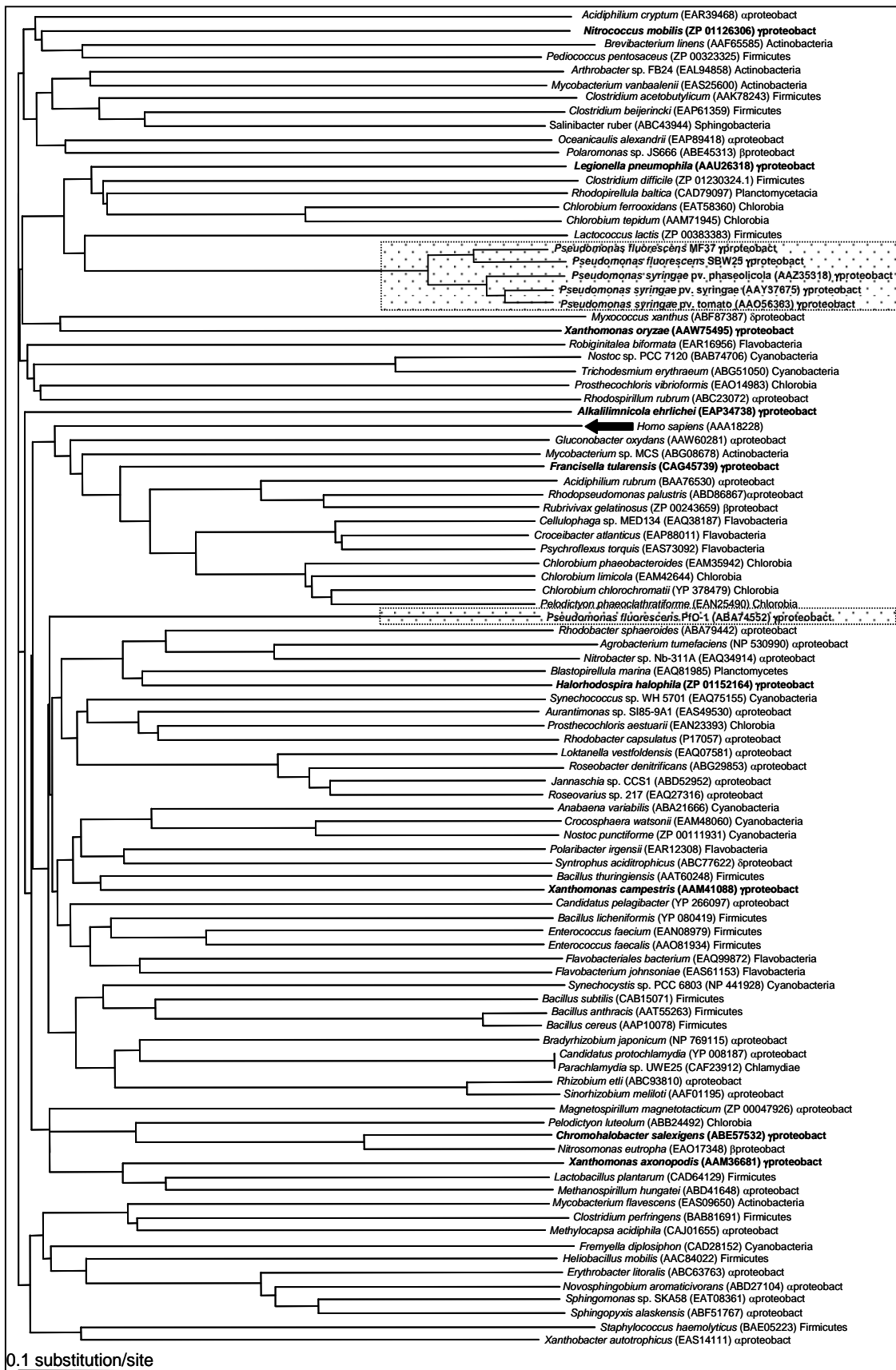


Figure 15 : Cladogramme de la protéine TPO bactérienne chez 97 espèces ou souches bactériennes (Chapalain et al, 2009).

Dans le cadre de la thèse de Charlène Leneveu, nous avons recherché les conditions dans lesquelles le gène *tspo* est transcrit chez *P. fluorescens* Pf0-1, et évalué les fonctions potentielles de la protéine TSPO chez cette bactérie. De nombreuses tentatives et stratégies ont été mises en place pour essayer d'obtenir un mutant de délétion du gène *tspo* mais elles se sont avérées infructueuses. Ce résultat était surprenant puisque des mutants du gène *tspo* avaient pu être construits chez d'autres organismes comme la mousse *Physcomitrella patens* (Lehtonen et al, 2012), ou *Arabidopsis thaliana* (Frank et al, 2007). Nous avons donc émis l'hypothèse que la présence du gène *tspo* pourrait être nécessaire chez *P. fluorescens* Pf0-1, et que ce gène ne pourrait être éliminé que dans certaines conditions environnementales restant à définir. Une approche *in silico* a donc été mise en œuvre pour obtenir des informations sur la structure et fonction potentielles de TSPO chez *P. fluorescens* Pf0-1. Cette étude a montré que chez *P. fluorescens* Pf0-1, tout comme chez la souche MF37 (Chapalain et al, 2009), la protéine est insérée dans la membrane cytoplasmique, le domaine N-terminal étant tourné vers le périplasme et l'extrémité C-terminale étant orientée vers le cytoplasme (**Article 8, Leneveu-Jenvrin et al, 2014, Figure 1**). Des motifs de liaison potentiels pour le cholestérol (ligand naturel) et le PK11195 (ligand artificiel) de la protéine mitochondriale ont aussi été retrouvés sur la protéine TSPO de *P. fluorescens* Pf0-1 (**Article 8, Leneveu-Jenvrin et al, 2014, Figure 2**). L'utilisation du logiciel STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, Franceschini et al, 2013) a permis de rechercher des partenaires potentiels susceptibles d'interagir avec la protéine TSPO, et de suggérer ainsi des fonctions potentielles de cette protéine dans des mécanismes vitaux de réponse de la bactérie aux stress oxydatif, la régulation de l'homéostasie du fer, le transport des porphyrines, la signalisation et la transduction de signaux, l'intégrité de la membrane et la virulence (**Article 8, Leneveu-Jenvrin et al, 2014, Figure 3**).

L'environnement génomique de TSPO chez *P. fluorescens* Pf0-1 a ensuite été étudié. Nous avons montré par qRT-PCR et par l'utilisation de fusions transcriptionnelles, que *tspo* est co-transcrit avec le gène Pfl01_2810 qui code pour une histidine kinase hybride (HyHK), suggérant l'existence d'une structure opéronique pour ces deux gènes mais aussi probablement un lien fonctionnel entre TSPO et HyHK. Cet opéron est exprimé de façon transitoire pendant la croissance bactérienne en milieu LB à 28°C. L'expression augmente rapidement en tout début de la phase exponentielle de croissance puis diminue rapidement.

L'activité transcriptionnelle varie aussi avec la température, elle est nulle à 18°C et très largement augmentée à 32°C, suggérant une régulation par le facteur sigma RpoH, généralement activé en réponse au stress thermique. En cas d'hyperosmolarité (NaCl, sucrose), ou en présence de D-cyclosérine, l'activité transcriptionnelle est très largement augmentée, engendrant un stress pariétal et une régulation par le facteur sigma AlgU *via* RpoH ([Article 9, Leneveu-Jenvrin et al, 2015](#)). L'utilisation de ligands de la TSPO mitochondriale tel que le cholestérol, qui est impliqué dans la fluidité membranaire, engendre également une augmentation de la transcription de *tspo*, ainsi que d'autres ligands Eucaryotes tels que le PK11195 ou la protoporphyrine IX suggérant une conservation fonctionnelle entre la protéine TSPO bactérienne de *P. fluorescens* Pf0-1 et son orthologue Eucaryote.

H) Participation à d'autres travaux de recherche (en collaboration)

Belguesmia et al, 2011

[Res Microbiol](#). 2011 Feb-Mar;162(2):159-63. doi: 10.1016/j.resmic.2010.09.019. Epub 2010 Oct 28.

Growing insights into the safety of bacteriocins: the case of enterocin S37.

[Belguesmia Y¹](#), [Madi A](#), [Sperandio D](#), [Merieau A](#), [Feuilloley M](#), [Prévost H](#), [Drider D](#), [Connil N](#).

+ Author information

Abstract

Very few studies have been reported on the cytotoxicity and impact of bacteriocins, and especially enterocins, upon eukaryotic cells. In order to gain more information on the safety of bacteriocins, we focused this study on enterocin S37, a bacteriocin produced by *Enterococcus faecalis* S37. We observed dose-dependent cytotoxicity toward undifferentiated Caco-2/TC7 cells. Moreover, no significant effect on differentiated monolayer Caco-2/TC7 and no apoptotic features were observed when cells were treated with 10 µg/ml of enterocin S37. The results obtained indicate possible safe use of enterocin S37 in the gastrointestinal tract of animals to prevent pathogen invasion and/or infection.

Morin et al, 2011

[Antimicrob Agents Chemother](#). 2011 Mar;55(3):997-1007. doi: 10.1128/AAC.01217-10. Epub 2010 Dec 28.

Mechanism of bactericidal activity of microcin L in *Escherichia coli* and *Salmonella enterica*.

[Morin N¹](#), [Lanneluc I](#), [Connil N](#), [Cottenceau M](#), [Pons AM](#), [Sablé S](#).

+ Author information

Abstract

For the first time, the mechanism of action of microcin L (MccL) was investigated in live bacteria. MccL is a gene-encoded peptide produced by *Escherichia coli* LR05 that exhibits a strong antibacterial activity against related Enterobacteriaceae, including *Salmonella enterica* serovars Typhimurium and Enteritidis. We first subcloned the MccL genetic system to remove the sequences not involved in MccL production. We then optimized the MccL purification procedure to obtain large amounts of purified microcin to investigate its antimicrobial and membrane properties. We showed that MccL did not induce outer membrane permeabilization, which indicated that MccL did not use this way to kill the sensitive cell or to enter into it. Using a set of *E. coli* and *Salmonella enterica* mutants lacking iron-siderophore receptors, we demonstrated that the MccL uptake required the outer membrane receptor Cir. Moreover, the MccL bactericidal activity was shown to depend on the TonB protein that transduces the proton-motive force of the cytoplasmic membrane to transport iron-siderophore complexes across the outer membrane. Using carbonyl cyanide 3-chlorophenylhydrazone, which is known to fully dissipate the proton-motive force, we proved that the proton-motive force was required for the bactericidal activity of MccL on *E. coli*. In addition, we showed that a primary target of MccL could be the cytoplasmic membrane: a high level of MccL disrupted the inner membrane potential of *E. coli* cells. However, no permeabilization of the membrane was detected.

Fito-Boncompete et al, 2011

[Infect Immun](#). 2011 Mar;79(3):1176-86. doi: 10.1128/IAI.00850-10. Epub 2010 Dec 28.

Full virulence of *Pseudomonas aeruginosa* requires OprF.

[Fito-Boncompete L¹](#), [Chapalain A](#), [Bouffartigues E](#), [Chaker H](#), [Lesouhaitier O](#), [Gicquel G](#), [Bazire A](#), [Madi A](#), [Connil N](#), [Véron W](#), [Taupin L](#), [Toussaint B](#), [Cornelis P](#), [Wei Q](#), [Shioya K](#), [Déziel E](#), [Feuilloley MG](#), [Orange N](#), [Dufour A](#), [Chevalier S](#).

+ Author information

Abstract

OprF is a general outer membrane porin of *Pseudomonas aeruginosa*, a well-known human opportunistic pathogen associated with severe hospital-acquired sepsis and chronic lung infections of cystic fibrosis patients. A multiphenotypic approach, based on the comparative study of a wild-type strain of *P. aeruginosa*, its isogenic oprF mutant, and an oprF-complemented strain, showed that OprF is required for *P. aeruginosa* virulence. The absence of OprF results in impaired adhesion to animal cells, secretion of ExoT and ExoS toxins through the type III secretion system (T3SS), and production of the quorum-sensing-dependent virulence factors pyocyanin, elastase, lectin PA-1L, and exotoxin A. Accordingly, in the oprF mutant, production of the signal molecules N-(3-oxododecanoyl)-L-homoserine lactone and N-butanoyl-L-homoserine lactone was found to be reduced and delayed, respectively. *Pseudomonas* quinolone signal (PQS) production was decreased, while its precursor, 4-hydroxy-2-heptylquinoline (HHQ), accumulated in the cells. Taken together, these results show the involvement of OprF in *P. aeruginosa* virulence, at least partly through modulation of the quorum-sensing network. This is the first study showing a link between OprF, PQS synthesis, T3SS, and virulence factor production, providing novel insights into virulence expression.

Reffuveille et al, 2012

Infect Immun. 2012 May;80(5):1728-35. doi: 10.1128/IAI.08251-11. Epub 2012 Feb 13.

Involvement of peptidylprolyl cis/trans isomerases in *Enterococcus faecalis* virulence.

Reffuveille F¹, Connil N, Sanquinetti M, Posteraro B, Chevalier S, Auffray Y, Rince A.

[+ Author information](#)

Abstract

Peptidylprolyl cis/trans isomerases (PPIases) are enzymes involved in protein folding. Analysis of the genome sequence of *Enterococcus faecalis* V583 allowed for identification of 3 PPIases carrying genes. *ef2898* encodes an intracellular PPIase which was not shown to be important for the *E. faecalis* stress response or virulence. The other two PPIases, the parvulin family rotamase EF0685 and the cyclophilin family member EF1534, are expected to be surface-exposed proteins. They were shown to be important for virulence and resistance to NaCl. A Δ *ef0685* Δ *ef1534* mutant was also more resistant to oxidative stress, was able to grow under a high manganese concentration, and showed altered resistance to ampicillin and quinolone antibiotics.

Gicquel et al, 2013

PLoS One. 2013 Nov 18;8(11):e80407. doi: 10.1371/journal.pone.0080407. eCollection 2013.

The extra-cytoplasmic function sigma factor sigX modulates biofilm and virulence-related properties in *Pseudomonas aeruginosa*.

Gicquel G¹, Bouffartiques E, Bains M, Oxaran V, Rosay T, Lesouhaitier O, Connil N, Bazire A, Maillot O, Bénard M, Cornelis P, Hancock RE, Dufour A, Feuilloley MG, Orange N, Déziel E, Chevalier S.

[+ Author information](#)

Abstract

SigX, one of the 19 extra-cytoplasmic function sigma factors of *P. aeruginosa*, was only known to be involved in transcription of the gene encoding the major outer membrane protein OprF. We conducted a comparative transcriptomic study between the wildtype H103 strain and its sigX mutant PAOSX, which revealed a total of 307 differentially expressed genes that differed by more than 2 fold. Most dysregulated genes belonged to six functional classes, including the "chaperones and heat shock proteins", "antibiotic resistance and susceptibility", "energy metabolism", "protein secretion/export apparatus", and "secreted factors", and "motility and attachment" classes. In this latter class, the large majority of the affected genes were down-regulated in the sigX mutant. In agreement with the array data, the sigX mutant was shown to demonstrate substantially reduced motility, attachment to biotic and abiotic surfaces, and biofilm formation. In addition, virulence towards the nematode *Caenorhabditis elegans* was reduced in the sigX mutant, suggesting that SigX is involved in virulence-related phenotypes.

8) PROJETS DE RECHERCHE

8-1) PROJETS A COURT TERME

Projet 1 : Effet de l'Épinephrine sur la physiologie de bactéries pathogènes, commensales et probiotiques et identification des senseurs éventuels.

Ce projet a été intégré à une demande de financement COS-SESA (GRR FEDER-FUI), Volet 1 : Evaluation des risques émergents, action 5 « étude Probiot ».

*** Identification du senseur à l'épinephrine chez *Pseudomonas***

Dans le cadre de la fin de thèse de Kelly Biaggini et du master 2 de Valérie Borrel, nous avons commencé à étudier les effets de l'épinéphrine sur *P. aeruginosa*, mais aussi sur *Enterococcus faecalis*, qui est un autre germe présent au niveau de l'intestin. Comme cité précédemment, des récepteurs à l'épinéphrine homologues de Qsec ont été mis en évidence par analyse bioinformatique chez plus d'une vingtaine de pathogènes de l'homme ou des plantes (Rasko et al, 2008 ; Curtis et al, 2014), mais rien n'a été décrit pour l'instant chez les bactéries à gram positives comme *E. faecalis*. Chez *P. fluorescens* et *P. aeruginosa*, il semble exister un homologue de Qsec (senseur de l'épinéphrine/norépinéphrine d'*E. coli*), ceci pourrait permettre d'expliquer l'augmentation de la virulence observée lorsque ces bactéries étaient exposés à l'épinéphrine mais il faut maintenant confirmer que c'est bien ce récepteur qui intervient dans la reconnaissance de l'épinéphrine chez ces bactéries. Un inhibiteur, le LED209, ou [N-phenyl-4-[[[(phenylamino) thioxomethyl]amino]-benzenesulfonamide] peut-être utilisé pour bloquer spécifiquement Qsec, le récepteur bactérien des catécholamines (Rasko et al, 2008 ; Curtis et al, 2014). Il a été utilisé avec succès chez *Vibrio harveyi* pour bloquer l'augmentation de la mobilité bactérienne et la formation de biofilm par la norépinephrine et la dopamine (Yang et al, 2014). Nous allons tester aussi LED209 chez *P. fluorescens* et *P. aeruginosa*. Chez *E. coli* et *Salmonella enterica*, en plus d'être un senseur à l'épinephrine/norépinéphrine, Qsec est un senseur de l'autoinducteur 3 (AI-3), une molécule du *quorum sensing* impliquée dans la modulation de la virulence (Moreira et al, 2010).

Des fusions transcriptionnelles contenant différents gènes impliqués dans le *quorum sensing* et la formation de biofilm étant disponibles pour *P. aeruginosa* au laboratoire, nous allons rechercher quels gènes peuvent être activés par un traitement avec l'épinéphrine.

*** Effet de l'épinephrine sur les bactéries commensales et probiotiques**

Alors que l'action de l'épinéphrine/norépinéphrine sur la virulence de certains pathogènes commence à être bien caractérisée, leur effet sur les bactéries commensales et surtout sur les probiotiques est complètement inconnu. *E. faecalis* semble être un bon modèle pour cette étude car il existe dans cette espèce à la fois des souches pathogènes, des souches commensales et des souches utilisées en tant que probiotiques. Ce projet pourrait permettre de comparer l'effet de l'épinéphrine sur la virulence des souches d'*E. faecalis* pathogènes et leur action sur les souches d'*E. faecalis* commensales et probiotiques et d'établir éventuellement une sorte de risques/bénéfices de l'exposition à cette molécule de la population globale d'*E. faecalis* au niveau intestinal et plus généralement au niveau de l'organisme humain.

Pour ce projet, une collaboration pourrait être envisagée avec le Dr Pascal Serror, INRA Micalis, Jouy en Josa ; le Pr Alain Rincé, UR Risques microbiens, Caen et le Pr Djamel Drider, Laboratoire ProBioGEM, Polytech, Lille.

Projet 2 : Analyse du microbiote intestinal associé à l'anorexie mentale

Ce projet a obtenu un financement GRR IRIB 2014 (10k€)

Objectifs : Caractériser les bactéries intestinales majoritairement présentes chez les patients anorexiques et évaluer si l'anorexie mentale est associée à une dysbiose

*** Résumé du projet**

Le microbiote intestinal est un écosystème complexe dont la composition est influencée par différents facteurs tel que l'âge, le régime alimentaire, des facteurs génétiques, mais aussi la présence d'antibiotiques ou de probiotiques. Les études métagénomiques ont permis de découvrir que plus de 80% des bactéries hébergées par notre intestin ne sont pas cultivables. Cependant, de nombreux biais liés aux méthodes d'extraction, au choix des amorces ont été rapportés, pouvant conduire à des résultats différents à partir du même

échantillon en fonction de la technique utilisée, ou des résultats contradictoires suivant les équipes de recherche ayant effectué les travaux. Plus récemment, la culturomique bactérienne a été proposée comme un outil de choix pour l'analyse du microbiote intestinal (Lagier et al, 2012). Elle est basée sur l'utilisation d'un grand nombre de conditions de culture couplée à une méthode d'identification rapide par spectrométrie de masse MALDI-TOF ou par amplification et séquençage de l'ARN 16S pour les colonies non identifiées. Contrairement au microbiote des personnes obèses ou diabétiques (Turnbaugh et al, 2009, Bekkering et al, 2013), la flore intestinale des patients anorexiques a été très peu étudié jusqu'à présent (Pfleiderer et al, 2013). On peut cependant supposer que la modification de la sécrétion hormonale (sérotonine, leptine, ghreline, PYY..) qui a été décrite chez les patients anorexiques (Kaye et al, 1991, Singhal et al, 2014) ainsi que la baisse de disponibilité en nutriments pourrait affecter la composition du microbiote et/ou que ce microbiote pourrait aussi agir sur l'axe intestin/cerveau et peut être moduler la satiété et la prise alimentaire. Dans ce contexte, notre projet propose de caractériser par culturomique/MALDI-TOF les bactéries associées au phénotype anorexie. Les modifications du microbiote intestinal des fèces d'un modèle de rats anorexiques (ABA, activity-based anorexia model, Gutierrez, 2013) soumis à une restriction alimentaire entraînant chez l'animal une activité de course excessive, seront comparées à des données physiopathologiques issues de l'analyse des fèces de patients anorexiques.

*** Intérêt scientifique du sujet**

Les troubles du comportement alimentaire voient augmenter leur fréquence dans tous les pays du monde. En France, l'anorexie mentale toucherait environ 1,5 % des personnes de sexe féminin, entre 15 et 35 ans soit environ 230 000 personnes. Il s'agit donc d'un véritable problème de santé publique. Alors que la flore intestinale associée à l'obésité a été en partie caractérisée, très peu d'études ont été réalisées concernant des patients anorexiques et sur la modification du microbiote suite à une restriction alimentaire.

*** Conséquences attendues**

A court terme : Mettre en évidence l'émergence de certaines espèces, genres ou groupes bactériens suite à une restriction alimentaire.

A plus long terme : Une meilleure connaissance du microbiote associé à l'anorexie mentale pourrait permettre d'aider à la sélection de probiotiques pour compléter les apports nutritionnels du patient et restaurer sa flore intestinale.

Pour ce projet, une collaboration est en cours avec l'unité INSERM U1073, Nutrition, inflammation et dysfonction de l'axe intestin-cerveau, Rouen, avec le Pr Pierre Déchelotte, Pr Serguei Fetissov, Dr Moïse Coeffier et le Dr Rachel Marion-Letellier.

8-2) PROJET A LONG TERME

Effet de l'administration orale de bactéries probiotiques sur l'axe intestin-cerveau-peau et sur le microbiote cutané

La réalisation de ce projet est lié à l'obtention d'une bourse de thèse ou d'un financement industriel ou académique pour recruter un post-doctorant.

*** Résumé du projet**

Deux dermatologues, John H. Stokes et Donald ont proposé pour la première fois il y a plus de 70 ans, un lien entre l'intestin, la dépression, l'anxiété et les maladies cutanées. Ils ont émis l'hypothèse que l'état émotionnel pourrait altérer le microbiote intestinal, augmenter la perméabilité intestinale et contribuer à l'inflammation systémique. De nombreux aspects de cette théorie de l'axe intestin-cerveau-peau ont été étudiés récemment mais nécessitent des recherches complémentaires pour en décrypter les mécanismes moléculaires.

L'objectif de ce projet est d'évaluer l'effet de l'administration orale de bactéries probiotiques, sur le microbiote cutané et l'axe intestin-cerveau-peau. Des modèles *in vitro* et *in vivo*, ainsi qu'une étude clinique seront entrepris pour mieux comprendre comment les bactéries probiotiques interagissent avec l'axe intestin-cerveau-peau. La capacité d'un traitement oral par des probiotiques à modifier le microbiote cutané sera aussi examinée.

*** Expérience antérieure acquise sur le sujet par le laboratoire**

Comme décrit précédemment dans ce manuscrit, le LMSM travaille depuis plusieurs années sur la réponse bactérienne à son microenvironnement et sur le dialogue bactérie-hôte. Ses compétences sur le microbiote cutané sont reconnues par le pôle de compétitivité Cosmetic Valley et au niveau international.

Après avoir étudié l'effet de molécules eucaryotes sur la virulence de bactéries pathogènes, nous souhaitons aujourd'hui aborder la question du rôle des probiotiques pris par voie orale dans l'homéostasie cutanée (incluant le microbiote, l'épithélium, et le système immunitaire). La santé de la peau étant influencée par l'axe intestin-cerveau, le LMSM souhaite poursuivre sa collaboration avec l'unité Inserm U1073 sur ce projet afin d'avoir une approche globale de l'effet des probiotiques sur l'axe intestin-cerveau-peau. L'unité Inserm U1073 a une renommée internationale sur la physiologie intestinale et les dysfonctions intestin-cerveau. Ce laboratoire dispose de modèles animaux et de données cliniques qui pourraient permettre de compléter par une approche *in vivo* les études *in vitro* qui seront menées par le LMSM.

*** Contexte scientifique (état de l'art avec références bibliographiques)**

Une grande diversité de microorganismes, bactéries, champignons, virus, et arthropodes colonisent la peau humaine. Les pathologies cutanées telles que l'acné, la dermatite atopique, la rosacée et le psoriasis sont le plus souvent multifactorielles et associent des prédispositions génétiques, des dérèglements immunitaires, des perturbations physiologiques (surproduction de sébum, hyperkératinisation), et des modifications du microbiote cutané (Chandra et al, 2015 ; Grice, 2014). Un traitement local ou par voie orale, d'antibiotiques ou d'anti-inflammatoires peut permettre d'améliorer et/ou de faire disparaître certains symptômes cutanés et a donc été utilisé couramment pendant des décennies, comme thérapie contre les problèmes de peau. Ainsi dans le cas de l'acné, le traitement consiste en l'éradication de la bactérie *Propionibacterium acnes*. Cependant, avec l'apparition de bactéries résistantes, les traitements antibiotiques sont de moins en moins efficaces (Patel et al, 2010). Il est donc nécessaire de développer des méthodes alternatives de traitement. Ainsi les probiotiques, microorganismes vivants qui consommés en quantité adéquate produisent un effet bénéfique pour la santé, ont connu un essor remarquable au cours des vingt dernières années. Le traitement des pathologies cutanées par les

probiotiques peut être envisagé par voie orale mais aussi par administration topique. Dès 1912, il avait été observé que *Lactobacillus bulgaricus* peut aider dans le traitement local de l'acné (Peyri, 1912) mais les mécanismes d'action des probiotiques n'ont commencé à être étudiés que bien plus tard. Ainsi, en 1999, il a été montré que *Streptococcus thermophilus*, une espèce présente dans les yaourts permet d'augmenter la production de céramide lorsqu'elle est appliquée sur la peau (Di Marzio, 1999) et certains céramides ont une activité anti-inflammatoire et anti-microbienne contre *P. acnes* (Pavicic et al, 2007). *Bifidobacterium longum* et *Lactobacillus paracasei* CNCM I-2116 (ST11) peuvent réduire l'inflammation de la peau médiée par la substance P (Gueniche et al, 2010). L'application d'une lotion probiotique contenant *Enterococcus faecalis* pendant 8 semaines a permis de diminuer les lésions inflammatoires de 50% par rapport au placebo (Kang et al, 2009).

Des probiotiques pris par voie orale semblent également avoir un effet bénéfique sur la peau et plus généralement sur l'axe cerveau-intestin-peau (the brain-gut-skin theory). Dès 1930, Stokes et Pillsbury ont émis l'hypothèse que l'état émotionnel (nervosité, dépression, anxiété), pourrait altérer les fonctions gastro-intestinales, et modifier le microbiote intestinal qui à son tour provoquerait une inflammation locale et systémique pouvant conduire aux problèmes cutanés (Bowe et Logan, 2011). Une croissance trop importante des bactéries intestinales est fortement associée à la dépression et l'anxiété (Addolorato et al, 2008) alors que leur éradication permet d'améliorer l'état émotionnel des patients (Pimentel et al, 2000). Une étude récente montre que des probiotiques pris par voie orale peuvent réguler la libération de cytokines inflammatoires au niveau de la peau (Hacini-Rachinel, 2009). L'effet des probiotiques administrés par voie orale sur le microbiote intestinal a été en partie étudié (de Moreno de LeBlanc et LeBlanc, 2014), mais pas leur éventuel impact sur la modification du microbiote cutané. En 2014, Ishii et al ont montré que l'administration orale de *Bifidobacterium breve* permet d'atténuer la perturbation de la barrière épithéliale de la peau des souris qui a été préalablement altérée par un traitement UV (Ishii et al, 2014). Ces auteurs émettent l'hypothèse que la bactérie apportée par voie orale pourrait interagir avec certains facteurs de l'intestin de l'hôte, et que des médiateurs, par exemple des cytokines ou des constituants bactériens seraient transportés de l'intestin vers la peau via le système sanguin. Les phénols produits par les bactéries intestinales notamment pourraient s'accumuler dans d'autres organes et participer aux troubles cutanés (Iizuka et al, 2009).

Par ailleurs, alors que plusieurs molécules intestinales sont capables d'augmenter la croissance et ou la virulence de bactéries pathogènes (Dagorn et al, 2013 ; Madi et al, 2013), leurs effets sur les bactéries probiotiques ne sont pas documentés et nécessitent d'être évalués pour savoir si elles stimulent ou au contraire freinent leur colonisation et leur activité bénéfique intestinale et extra-intestinale. Certaines souches de *Lactobacillus* et *Bifidobacterium* sont connues comme productrices de GABA, sérotonine, catécholamines, acétylcholine et ont un effet sur le cerveau (Barrett et al, 2012 ; Sudo, 2014 ; Wall et al, 2014) mais on ne connaît pas le rôle ou l'effet de ces molécules chez les bactéries probiotiques.

Dans ce contexte, l'objectif de ce projet sera de rechercher l'effet des bactéries probiotiques sur l'axe intestin-cerveau-peau et d'analyser les modifications éventuelles du microbiote cutané après traitement oral par un probiotique, en s'appuyant sur des modèles in vitro et in vivo et si possible également des études cliniques, en associant les compétences du LMSM et celles de l'unité Inserm U1073.

*** Originalité et caractère innovant des recherches**

Jusqu'à présent la très grande majorité des travaux sur les probiotiques s'est focalisée sur leur effet bénéfique au niveau de l'intestin, l'effet de leur administration locale au niveau de la peau, et plus récemment leur interaction avec le cerveau par la production de molécules neuromodulatrices (bactéries psychobiotiques). Ce projet pourrait permettre d'avoir une approche globale des interactions des probiotiques avec l'axe intestin-cerveau-peau, en recherchant d'une part si certaines molécules eucaryotes sont capables de moduler les propriétés bénéfiques des probiotiques et en analysant d'autre part la modification potentielle du microbiote cutané suite à un traitement par voie orale avec le probiotique.

*** Approches méthodologiques**

A) Effet des neurotransmetteurs sur les probiotiques

Cette partie de l'étude pourra être réalisée sur des cellules intestinales (Caco-2) et des kératinocytes (Hacat). Un ou plusieurs probiotiques seront sélectionnés tels que *Lactobacillus casei*, *Lactobacillus salivarius*, *Enterococcus faecalis*...

L'effet des neurotransmetteurs (epinephrine/norepinephrine, sérotonine, GABA, substance P) sur les principales propriétés des probiotiques sera étudié, notamment l'adhésion

bactérienne, l'induction de la production de cytokines anti-inflammatoires, le renforcement de la barrière épithéliale, la production de peptides antimicrobiens, grâce à des outils biochimiques, génétiques et microscopiques.

Ces expériences permettront de mettre en évidence des mécanismes impliqués dans les signaux cerveau-probiotique-intestin et cerveau-probiotique-peau.

B) Effet des probiotiques pris par voie orale sur l'homéostasie cutanée de la souris

Des souris seront gavées pendant une semaine avec un probiotique ou plusieurs probiotiques comme par exemple le mélange VSL#3 contenant 8 souches bactériennes (*Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*). A la fin du gavage J8, le microbiote cutané de la souris sera analysé et comparé au jour J0 du gavage. L'analyse du microbiote sera effectuée par Maldi-Biotyper et/ou métagénomique et techniques d'identification classique de microbiologie.

Les cytokines pro-inflammatoires et anti-inflammatoires et les défensines murines seront quantifiées par qPCR. L'état de la peau de la souris sera évalué par cornéométrie, spectroscopie Raman et microscopie confocale à l'aide de marqueurs spécifiques de l'épithélium.

C) Etude clinique : effet des probiotiques pris par voie orale sur le microbiote cutané et l'état de la peau

Cette partie du projet sera à redéfinir avec l'unité Inserm U1073. Une cohorte de patients ayant ou non des troubles inflammatoires digestifs et/ou cutanés, et devant recevoir un traitement probiotique par voie orale pourrait être sélectionnée. Le microbiote cutané des patients sera analysé à partir d'un prélèvement effectué par écouvillonnage au jour J0 du traitement probiotique et au jour Jn correspondant à la fin du traitement. La composition du microbiote sera analysée par Maldi-Biotyper et/ou métagénomique et techniques d'identification classique de microbiologie. L'état de la peau du patient sera évalué par le praticien hospitalier. Une enquête alimentaire établie avec la diététicienne pour connaître les habitudes du patient (consommation de yaourt, de laits ou produits fermentés..) permettra d'avoir des informations complémentaires pour cette étude.

*** Identification des avancées qui donneront lieu à publication**

L'étude fondamentale de l'effet des molécules eucaryotes sur les propriétés des probiotiques (modèle *in vitro*) pourra conduire au minimum à 2 publications suivant les probiotiques qui seront sélectionnés et les mécanismes moléculaires qui seront mis en évidence. L'analyse du microbiote de la souris, et des patients traités avec un ou plusieurs probiotiques devrait permettre également de rédiger 1 ou 2 publications.

*** Valorisations opérationnelles et scientifiques**

Les résultats obtenus sur les probiotiques au cours de ce projet permettront par la suite d'aider au choix rationnel pour leur utilisation et la mise à jour de certaines allégations nutritionnelles et thérapeutiques. Les découvertes scientifiques majeures seront présentées sous forme de conférences et posters dans des colloques nationaux et internationaux, et feront l'objet de publications dans des revues spécialisées de microbiologie, dermatologie, physiologie et/ou neurologie.

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-ANNEXES-

-Article 1-

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RESEARCH ARTICLE

Open Access

The clinical *Pseudomonas fluorescens* MFN1032 strain exerts a cytotoxic effect on epithelial intestinal cells and induces Interleukin-8 via the AP-1 signaling pathway

Amar Madi¹, Omar Lakhdari³, Hervé M Blottière³, Muriel Guyard-Nicodème¹, Karine Le Roux³, Anne Groboillot¹, Pascal Svinareff², Joel Doré³, Nicole Orange¹, Marc GJ Feuilloley¹, Nathalie Connil^{1*}

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Abstract

Background: *Pseudomonas fluorescens* is present in low number in the intestinal lumen and has been proposed to play a role in Crohn's disease (CD). Indeed, a highly specific antigen, I2, has been detected in CD patients and correlated to the severity of the disease. We aimed to determine whether *P. fluorescens* was able to adhere to human intestinal epithelial cells (IECs), induce cytotoxicity and activate a proinflammatory response.

Results: Behaviour of the clinical strain *P. fluorescens* MFN1032 was compared to that of the psychrotrophic strain *P. fluorescens* MF37 and the opportunistic pathogen *P. aeruginosa* PAO1. Both strains of *P. fluorescens* were found to adhere on Caco-2/TC7 and HT-29 cells. Their cytotoxicity towards these two cell lines determined by LDH release assays was dose-dependent and higher for the clinical strain MFN1032 than for MF37 but lower than *P. aeruginosa* PAO1. The two strains of *P. fluorescens* also induced IL-8 secretion by Caco-2/TC7 and HT-29 cells via the AP-1 signaling pathway whereas *P. aeruginosa* PAO1 potentially used the NF- κ B pathway.

Conclusions: The present work shows, for the first time, that *P. fluorescens* MFN1032 is able to adhere to IECs, exert cytotoxic effects and induce a proinflammatory reaction. Our results are consistent with a possible contribution of *P. fluorescens* in CD and could explain the presence of specific antibodies against this bacterium in the blood of patients.

Background

Pseudomonas aeruginosa is an opportunistic pathogen frequently emerging from the mucosa-associated intestinal microbiota, which can cause severe septicemia in immuno-compromised hosts. Several interaction mechanisms of *P. aeruginosa* with intestinal epithelial cells (IECs), especially adhesion and penetration, have been studied in detail [1-3]. Conversely, little attention has been given to other species of the same genus, like *Pseudomonas fluorescens*.

Pseudomonas fluorescens has long been considered as a psychrotrophic microorganism, unable to grow at temperatures over 32°C, however we have recently shown

that some strains isolated from a clinical environment are able to grow at or above 37°C [4]. *P. fluorescens* is a widespread gram-negative bacterium present in a variety of ecological niches such as refrigerated food products, soil, water [5] and in the digestive tract [6]. Interestingly, a highly specific antigen of *P. fluorescens*, designated as I2, was detected in the serum of 54% of the patients suffering from ileal Crohn's disease (CD) [7] and a direct link between the severity of the pathology and the level of circulating I2 antigen has been demonstrated [8]. Surprisingly, the proinflammatory potential of this bacterium or its interaction with the intestinal epithelium has never been investigated.

Several studies have focused on the mucosal immune response to pathogenic bacteria. Human IECs infected with pathogenic bacteria generally produce proinflammatory cytokines, such as interleukin (IL)-8 [9]. The

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latter has a chemotactic role and can recruit polymorphonuclear cells into the infected site and promote their infiltration of the epithelial layer infected by invasive or noninvasive bacteria [10,11]. IL-8 gene expression is regulated by two major transcriptional factors: nuclear factor kappa B (NF- κ B) and activator protein (AP)-1 [12]. NF- κ B has a pivotal role in the immune and inflammatory response, but also controls cell survival, proliferation and differentiation [13,14]. Recent works demonstrated that NF- κ B signaling is a critical element of the homeostatic immuno-inflammatory function in the gut. Indeed, epithelial NF- κ B preserves the integrity of the gut epithelial barrier and coordinates the antimicrobial actions of the innate and adaptive immune systems [15]. Nevertheless, hyperactivation of this transcription factor results in chronic inflammatory bowel diseases [16]. Activation of AP-1 is dependent on mitogen-activated protein kinases (MAPK) that are central in many physiological processes, including regulation of cytokine and stress responses and cytoskeletal reorganization [17,18].

P. fluorescens MFN1032 is a clinical strain recently isolated in our laboratory [19]. It displays hemolytic activity toward sheep erythrocytes [20,21], however, its infectious potential on human IECs is still unknown.

In the present study, we investigated adhesion and cytotoxic properties of *P. fluorescens* MFN1032 on Caco-2/TC7 and HT-29 cell lines in comparison to the psychrotrophic strain, *P. fluorescens* MF37 and the well-known opportunist pathogen *P. aeruginosa* PAO1. The proinflammatory potential of *P. fluorescens* MFN1032 was also evaluated by the measurement of IL-8 secretion on both Caco-2/TC7 and HT-29 cells, and analysis of NF- κ B and AP-1 activation using the reporter gene strategy.

Results

Adhesion to intestinal epithelial cells

The binding index of the clinical strain *P. fluorescens* MFN1032 on Caco-2/TC7 and HT-29 cells was determined after 5 h of incubation and compared to *P. fluorescens* MF37 and *P. aeruginosa* PAO1. The data presented in Figure 1 show that these bacterial strains adhere to both cell lines but the binding index was higher for Caco-2/TC7 (Figure 1A) than for HT-29 (Figure 1B).

P. aeruginosa PAO1 showed the highest adhesion potential on Caco-2/TC7 cells compared to *P. fluorescens* MF37 and *P. fluorescens* MFN1032. When the cells were infected with a 10^6 CFU or 10^8 CFU ml⁻¹ bacterial solution, the mean adhesion index of *P. aeruginosa* PAO1 reached 12.6 ± 2.6 or 32.1 ± 1.9 bacteria cell⁻¹, respectively, whereas the adhesion of *P. fluorescens* was quite similar for the two strains with 10.6 ± 0.5 or

18.1 ± 1.9 bacteria cell⁻¹ and 8.2 ± 0.6 or 19.8 ± 2 bacteria cell⁻¹ for MF37 and MFN1032, respectively.

The same experiment using HT-29 cells showed that the binding index of *P. aeruginosa* PAO1 remained the highest (7.1 ± 0.8 or 10.1 ± 1.0 bacteria cell⁻¹) but the index of *P. fluorescens* MFN1032 (4.3 ± 0.6 or 8.3 ± 1.6 bacteria cell⁻¹) was significantly higher than that of MF37 (1.4 ± 0.2 or 2.3 ± 0.5 bacteria cell⁻¹).

Cytotoxicity assay

The cytotoxic effect of *Pseudomonas* strains on Caco-2/TC7 and HT-29 cells was determined by quantification of lactate dehydrogenase (LDH) released in culture medium (Figure 2).

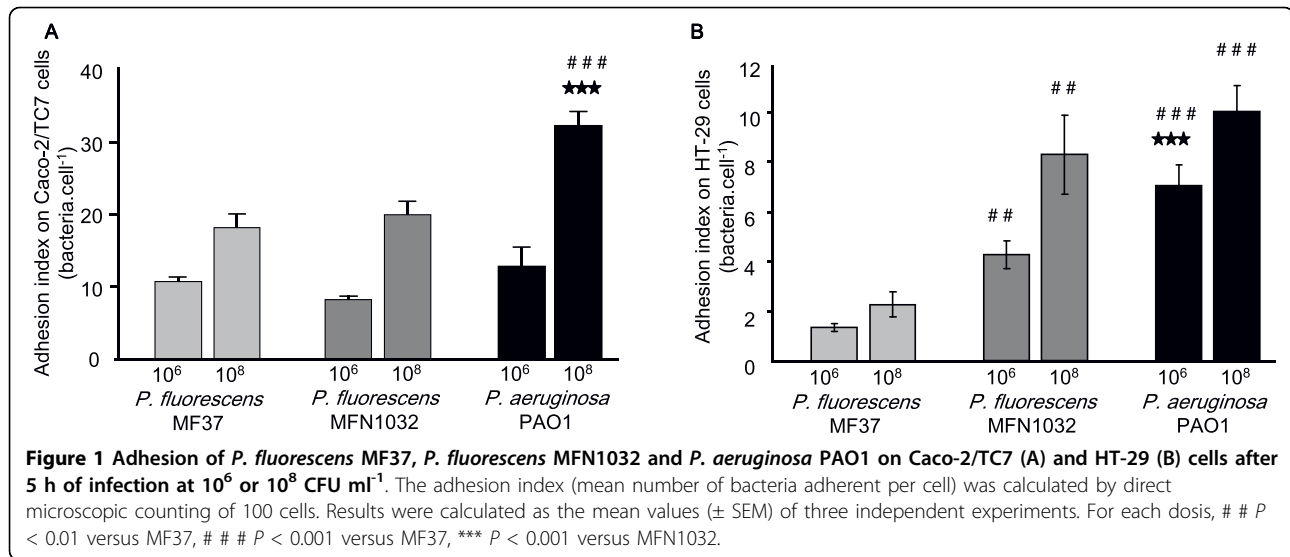
P. fluorescens MF37 exhibited the lowest cytotoxic activity (expressed as % of maximal LDH release) with only $7.8 \pm 1.9\%$ (at 10^6 CFU ml⁻¹) or $30 \pm 16.4\%$ (at 10^8 CFU ml⁻¹) of cell lysis after 24 h of infection on Caco-2/TC7 (Figure 2A) and $17.5 \pm 1.1\%$ (at 10^6 CFU ml⁻¹) or $22 \pm 2.0\%$ (at 10^8 CFU ml⁻¹) of cell lysis for HT-29 cells (Figure 2B). The cytotoxicity of MFN1032 was higher with $34 \pm 15.2\%$ or $74.7 \pm 4.6\%$ lysis for infection respectively with 10^6 or 10^8 CFU ml⁻¹ on Caco-2/TC7 and 33.2 ± 1.5 or $60.3 \pm 5.5\%$ lysis after infection with 10^6 or 10^8 CFU ml⁻¹ respectively on HT-29. *P. aeruginosa* PAO1 led to a total lysis of Caco-2/TC7 at the two bacterial concentrations tested and on HT-29, with infection rates of 10^6 or 10^8 CFU ml⁻¹, LDH release was $67.9 \pm 7.2\%$ or $85.6 \pm 3.4\%$ respectively. At the end of infection, Caco-2/TC7 and HT-29 cells were observed by light microscopy. Figure 3 shows the cell monolayers after infection with MOI of 100 (10^8 CFU ml⁻¹). When Caco-2/TC7 cells were infected with *P. fluorescens* MF37, a slight cell detachment was detectable while more cells were detaching after infection with MFN1032. Infection with *P. aeruginosa* PAO1 led to a complete disappearance of the organized Caco-2/TC7 and HT-29 monolayers.

Induction of IL-8 secretion

The bacterial proinflammatory effect was assessed by measuring IL-8 secretion. Compared to untreated cells, the three *Pseudomonas* strains induced significant stimulation of IL-8 secretion in both Caco-2/TC7 (Figure 4A) and HT-29 cells (Figure 4B). Mean values of IL-8 on HT-29 and Caco-2 in response to *P. fluorescens* MF37 and MFN1032 were similar for these two strains and it is noteworthy that IL-8 secretion was significantly increased in HT-29 compared to Caco-2 cells.

NF- κ B and AP-1 activation in Caco-2 and HT-29 reporter cell lines

To further explore the immuno-modulatory properties of *P. fluorescens* MFN1032, we tested the effects of this



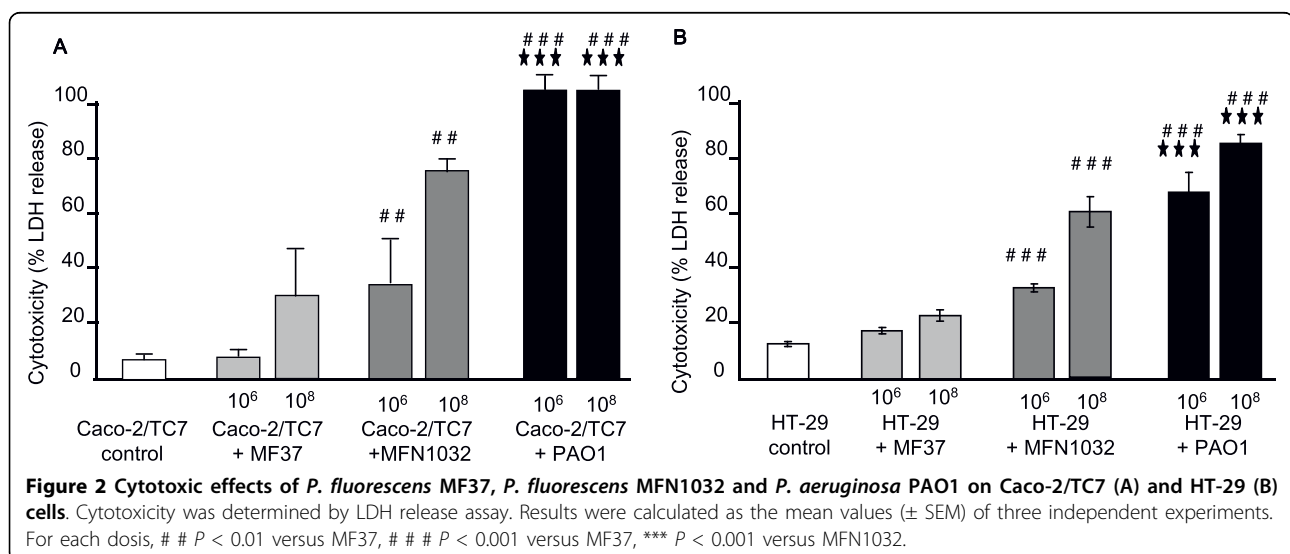
bacterium on NF- κ B or AP-1 activation using Caco-2 and HT-29 reporter cell lines. We observed that *P. aeruginosa* PAO1 stimulated NF- κ B activity by 2.5-fold over control in both Caco-2/ κ b-seap-7 and HT-29/ κ b-seap-25 reporter clones (Figure 5) while it had no effect on the AP-1 pathway (Figure 6). Interestingly, *P. fluorescens* MF37 and MFN1032 had an opposite effect. Indeed, none of these strains induced NF- κ B activation (Figure 5) whereas they both activated the AP-1 pathway by 2.2-fold over control in Caco-2/ap1-luc-1 and HT-29/ap1-luc-6 reporter clones (Figure 6).

Discussion

P. fluorescens is present at low level in the human gut and has been linked to Crohn's disease (CD) [7,8], however little is known about the potential interaction of

this bacterium with the intestinal mucosa. In the present paper, we aimed at determining its potential to adhere to IEC, to induce cell cytotoxicity and trigger a proinflammatory response. We selected two strains, a classical psychrotrophic strain (MF37) and a recently characterized clinical strain adapted to grow at 37°C (MFN1032). The behaviour of these bacteria was compared to that of the opportunistic pathogen *P. aeruginosa*.

Since adhesion and cytotoxicity to IECs are crucial events in the infection process, the three strains were tested on two epithelial cell lines. Except for adhesion, the two IECs models used in this study gave similar responses to the three strains of *Pseudomonas*. Indeed, a dose dependent adhesion of bacteria to Caco-2/TC7 and HT-29 cells was observed with the greatest effect obtained with the opportunistic pathogen



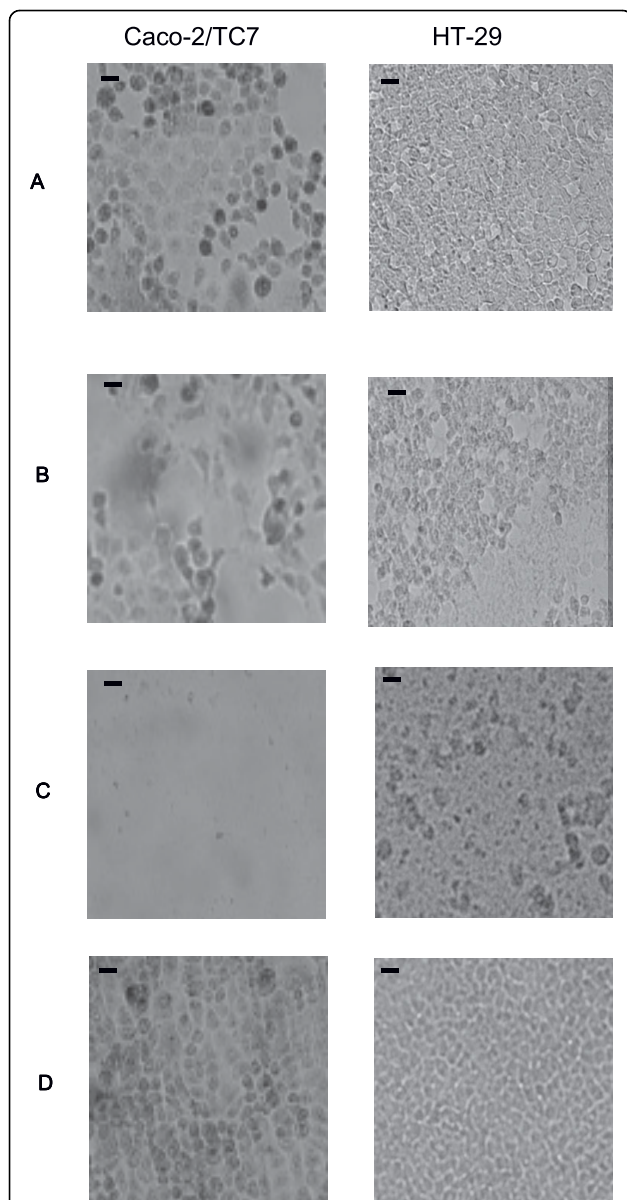


Figure 3 Effects of *P. fluorescens* MF37 (A), *P. fluorescens* MFN1032 (B) and *P. aeruginosa* PAO1 (C) on the morphological aspect of Caco-2/TC7 and HT-29 monolayers compared to a non-infected monolayer (D). The figure only shows the results obtained after 24 h of infection with a concentration of 10^8 CFU.ml⁻¹. Scale bar = 100 μ m.

P. aeruginosa. It is noteworthy that, compared to the psychrotrophic strain MF37, the clinical strain *P. fluorescens* MFN1032, which is adapted to develop at 37°C displayed statistically significant higher adhesion potential to HT-29 but not to Caco-2/TC7 cells. This observation suggests that the clinical strain may express a greater diversity of adhesion factors than MF37 and could explain, at least in part, the higher cytotoxicity effect of MFN1032.

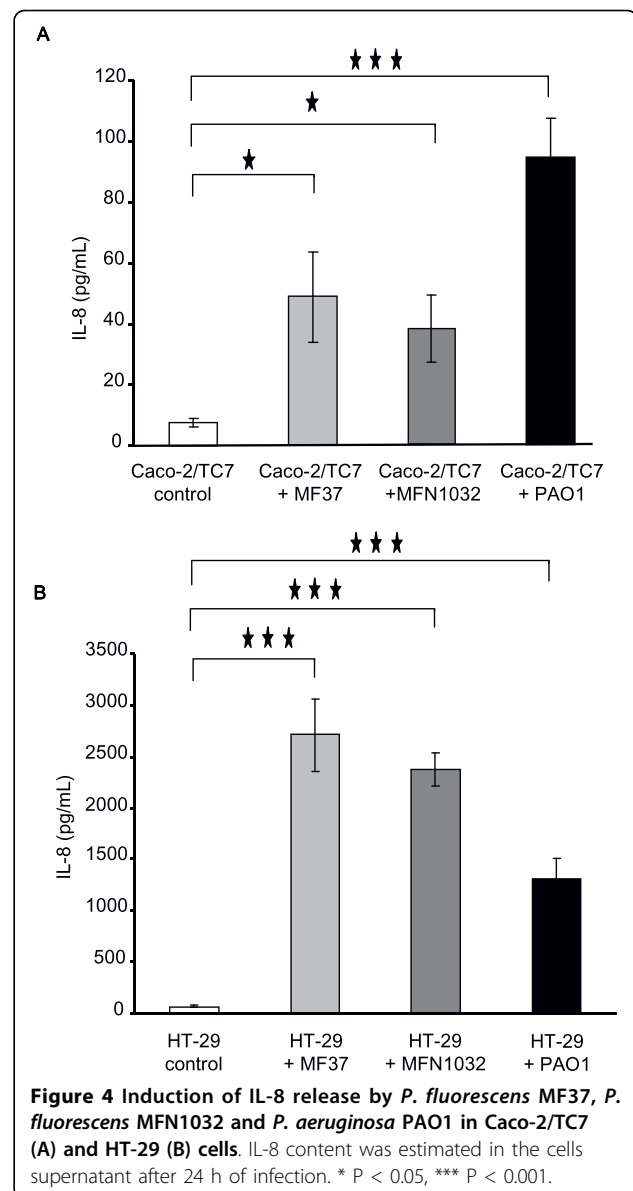
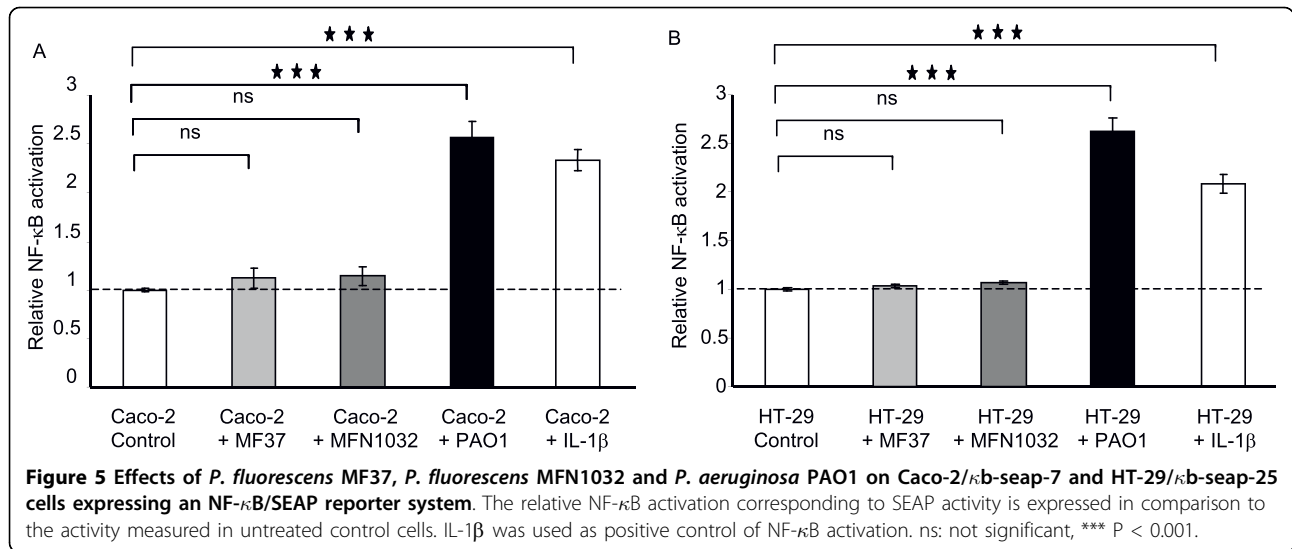


Figure 4 Induction of IL-8 release by *P. fluorescens* MF37, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 in Caco-2/TC7 (A) and HT-29 (B) cells. IL-8 content was estimated in the cells supernatant after 24 h of infection. * P < 0.05, *** P < 0.001.

Although differences exist between surface proteins expressed by Caco-2/TC7 and HT-29 cell lines in comparison to normal human IECs, our results support the hypothesis that *P. fluorescens* should be able to colonize the intestinal mucosa. *Pseudomonad* are rarely searched for and detected as fecal bacteria, and are usually considered as a sub-dominant population [22]. In addition, there is now ample evidence that the circulating bacterial population in the intestinal lumen is very different from the resident microbiota that comes in contact with the apical surface of the enterocytes and is tightly associated to the mucus/glycocalyx layer [23,24]. For an aerobic bacterium such as *P. fluorescens*, the best ecological niche should be at the vicinity of the epithelium, where oxygen concentration is the highest in the

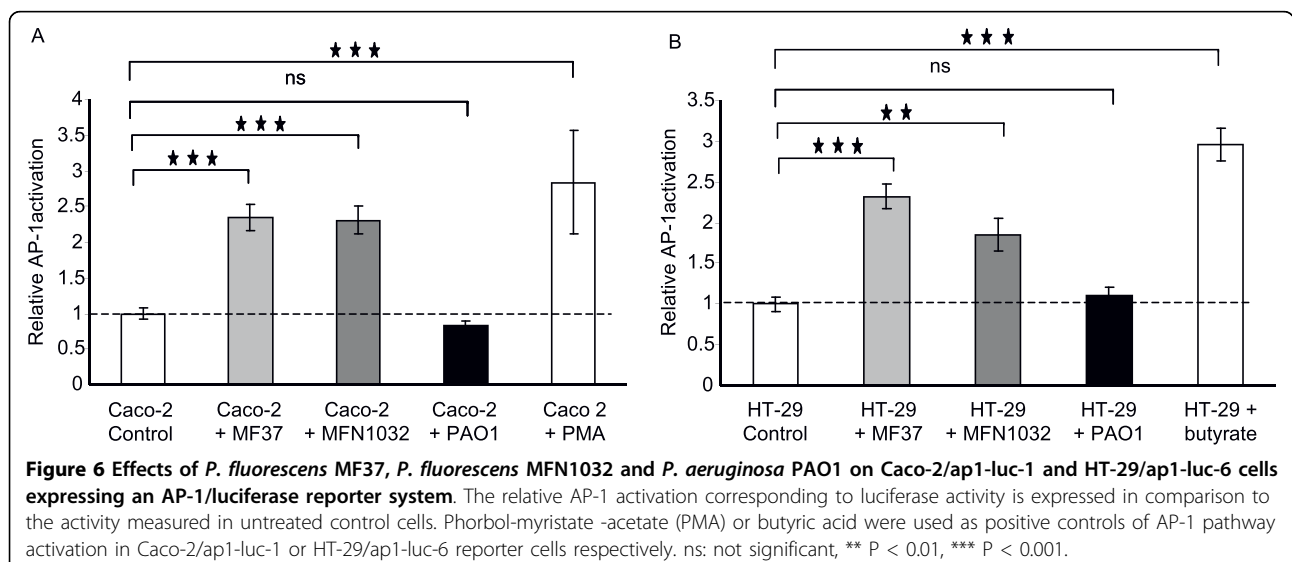


intestinal environment [25]. This is supported by the evidence showing that the *P. fluorescens*-specific I2 antigen sequence is systematically detected in ileal mucosa samples [7]. Moreover, in CD patients, there is a positive correlation between blood level of circulating anti-I2 antibodies and the severity of the disease [8] suggesting that the I2-producing bacteria, *i.e.* *P. fluorescens*, are in close contact with enterocytes and could contribute to CD pathogenesis.

The LDH release assay showed that the cytotoxicity of *P. fluorescens* on Caco-2/TC7 and HT-29 cells is lower than that of *P. aeruginosa*. This limited virulence of the *P. fluorescens* strains seems to be normal for a species that should be a resident in the intestine whereas *P. aeruginosa* is typically an opportunistic pathogen only detected in case of declared infection [26]. This

hypothesis is also in agreement with the hierarchy of the cytotoxic activity of the two tested strains of *P. fluorescens*, the clinical strain MFN1032 being more virulent than the environmental and psychrotrophic strain MF37. Bacterial cytotoxicity is a highly complex phenomenon combining the virulence of the prokaryote and the intrinsic sensitivity of the eukaryotic cell. In opposition to the present results, Chapalain *et al* found that the cytotoxic activity on glial cells was higher for *P. fluorescens* MF37 than MFN1032 [4]. These observations are in agreement with the work of Picot *et al* showing that in the case of *P. fluorescens*, the necrotic and apoptotic activities are not simply correlated to the adhesion potential of the strain [27].

In contrast to *P. aeruginosa*, the proinflammatory effect of *P. fluorescens* strains has not been elucidated.



In this study, we demonstrated that similarly to *P. aeruginosa*, *P. fluorescens* MFN1032 and MF37 exerted a direct proinflammatory effect on IECs as demonstrated by induction of IL-8 secretion. The homogenous proinflammatory response of IECs induced by the two *P. fluorescens* strains studied suggests a link between the proinflammatory properties and a common pathogenic factor of these strains. IL-8 gene expression is regulated by several signaling pathways including mainly NF- κ B and AP-1 transcription factors. Previous studies have shown that *P. aeruginosa* activates NF- κ B in mouse monocyte/macrophage cell line [28] and MAPK signaling pathways in lung epithelial cells [29], which in turn leads to the production of proinflammatory cytokines, such as IL-6, IL-8, and TNF- α (tumor necrosis factor alpha). In our study, the two *P. fluorescens* strains failed to activate the NF- κ B pathway in contrast to *P. aeruginosa*, however the two strains were able to activate AP-1 signaling, suggesting that the proinflammatory effect of these bacteria in IECs is linked to the activation of MAPK signaling pathways. The MAPK form a group of three pathways, including extracellular signal-regulated protein kinases (ERK1/2) and two stress-activated protein kinases designated p38 and JNK (c-jun N-terminal kinase) [30]. The activation of MAPK has been reported to be involved in response to infection by invasive bacteria, such as *Salmonella enterica* serovar typhimurium or *Listeria monocytogenes*, in IECs [31,32] or in macrophages [33]. Moreover, it has been shown that enteroadherent *Escherichia coli* activate this pathway and both bacterial attachment and secreted proteins might be implicated in cytokine responses [34]. *P. aeruginosa* as well as *P. fluorescens* contain multiple cell surface factors, including flagellin, pili, LPS, type III-mediated toxin secretion and quorum-sensing molecules which could interact with distinct epithelial membrane proteins, such as asialylated glycolipid receptors, toll like receptors (TLRs), or combinations of these proteins [28,35,36]. We have shown that purified flagellin strongly activated NF- κ B pathway in HT-29 and to a lower extent in Caco-2, whereas both cell lines poorly responded to LPS (Lakhdari et al, submitted manuscript). In contrast, purified flagellin and LPS do not activated the AP-1 pathway in the two cell lines (data not shown). Thus, we can conclude that *P. fluorescens* activated AP-1 pathway in Caco-2 and HT-29 independently of flagellin and LPS expression. Further investigations will be needed to identify the exact nature and function of *P. fluorescens* compounds responsible for MAPK activation in IECs.

Conclusions

P. fluorescens MFN1032, *P. fluorescens* MF37 and *P. aeruginosa* PAO1 were found to adhere to Caco-2/TC7 and HT-29 cells and the cytotoxicity towards these cell lines

was higher for the clinical strain MFN1032 than for MF37. We showed that the two strains of *P. fluorescens* induced IL-8 secretion by Caco-2/TC7 and HT-29 cells via the AP-1 signaling pathway whereas *P. aeruginosa* PAO1 potentially used the NF- κ B pathway. To our knowledge, this work is the first to demonstrate the interaction and the proinflammatory potential of *P. fluorescens* on IECs.

Methods

Cell culture

The human colon adenocarcinoma cell lines Caco-2/TC7 [37] and HT-29 were used between passages 10 and 35. Caco-2/TC7 cells were grown in Dulbecco's modified Eagle Minimal Essential Medium (Sigma) containing 20% foetal calf serum (FCS) supplemented with 2 mM of L-glutamine, 100 U ml⁻¹ each of penicillin and streptomycin and 1% non-essential amino acids at 37°C with 5% CO₂. HT-29 cells were grown in Dulbecco's modified Eagle Minimal Essential Medium (Sigma) containing 10% FCS supplemented with 2 mM of L-glutamine, 100 U ml⁻¹ each of penicillin and streptomycin at 37°C with 5% CO₂.

Bacterial strains and culture conditions

P. fluorescens MF37 is a rifampicin-resistant natural mutant of the strain MF0 (Biovar V), originally identified in crude milk [38]. *P. fluorescens* MFN1032, is a clinical biovar I strain collected in a hospital of Haute-Normandie (France) [4]. *P. aeruginosa* PAO1 was obtained from an international collection. Bacteria were grown overnight in ordinary nutrient broth (Merck) at 28°C for the two strains of *P. fluorescens* and at 37°C for *P. aeruginosa* PAO1. For adhesion and cytotoxicity assays, bacteria in stationary phase were harvested by centrifugation (5000 × g, 5 min, 20°C) and resuspended in antibiotic-free and serum-free cell culture media at densities of 10⁶ and 10⁸ CFU ml⁻¹, corresponding to a multiplicity of infection (MOI) of 1 and 100 respectively.

Adhesion assay

For adhesion assays, Caco-2/TC7 and HT-29 cells were seeded at a concentration of 1 × 10⁵ cells ml⁻¹ on coverslips coated with 50 μg ml⁻¹ poly-L-lysine and used at 80% confluence as recommended by Li et al [39]. Cells were incubated for 5 h with 1 ml of the bacterial suspensions. After incubation, the medium and non-adherent bacteria were removed by washing. Then, the coverslips were fixed with methanol (10 min), stained with Giemsa solution (20 min) and observed using an Axiovert S100TM light microscope (Zeiss). The adhesion index (mean number of bacteria adherent per cell) was determined by direct counting on a minimum of 100 cells following the technique of Darfeuille-Michaud et al [40].

Cytotoxicity assay

Confluent Caco-2/TC7 and HT-29 cells cultivated in 24-well culture plates were infected for 24 h with 1 ml of the bacterial suspensions. At the end of incubation, lactate dehydrogenase (LDH) present in the supernatant was measured in each well using the Cytotox 96^R enzymatic assay (Promega). LDH is a stable cytosolic enzyme released by eukaryotic cells and an overall indicator of necrosis. Caco-2/TC7 and HT-29 cells exposed to Triton X100 (0.9%) were used as a control of total release (100% LDH release). The background level (0% LDH release) was determined with serum free culture medium. The percentage of cytotoxicity was calculated following the manufacturer's instructions.

IL-8 ELISA

IL-8 assays were performed on confluent Caco-2/TC7 and HT-29 cells monolayers grown in 24-well culture plates. After 24 h of infection with the bacterial suspensions (MOI of 100), immunoreactive IL-8 protein levels in cell culture supernatant were quantified using an ELISA Quantikine kit (R&D systems) according to the manufacturer's protocol.

Construction of stable NF- κ B and AP-1 reporter cells

The NF- κ B reporter clones Caco-2/ κ b-seap-7 and HT-29/ κ b-seap-25 were obtained after a stable transfection of parental cells with the reporter plasmid pNiFty2-SEAP (Invivogen), which contains SEAP (secreted alkaline phosphatase) as reporter gene downstream of five repeats of the NF- κ B binding consensus.

The AP-1 reporter clones Caco-2/ap1-luc-1 and HT-29/ap1-luc-6 were obtained after a stable co-transfection of the reporter plasmid pAP-1-luc (Stratagen), which contains luciferase as reporter gene downstream of seven repeats of the AP-1 binding consensus, together with pTK-Hyg (Clontech) a hygromycin-based selection vector. Transfection of HT-29 was performed by lipofection using TFX-50 (Promega) according to the manufacturer's instructions while Caco-2 cells were transfected using the Amaxa Nucleofector system (Lonza).

Analysis of NF- κ B and AP-1 activation

For each experiment, reporter cells were seeded at 50 000 cells per well, into 96-well plates and pre-incubated 24 hours before adding live bacteria at an MOI of 100.

For NF- κ B activation assays, Caco-2/ κ b-seap-7 and HT-29/ κ b-seap-25 cells were incubated with live bacteria for 8 hours and IL-1 β (10 ng/ml) was used as a positive control. SEAP activity in the supernatant was measured using the Quanti-Blue reagent (Invivogen) using the manufacturer's protocol and quantified as OD at 655 nm.

For AP-1 activation assays, cells were incubated for 12 h with live bacteria. Phorbol-myristate -acetate (PMA, 1 μ M) or butyric acid (2 mM) was used as a positive control for Caco-2/ap1-luc-1 or HT-29/ap1-luc-6 reporter cells respectively. Luciferase activity was measured using the ONE-Glo™. Luciferase Assay System (Promega) according to the manufacturer's instructions and quantified as relative luminescence units (RLU).

All measurements were performed using a microplate reader (Infinite 200, Tecan).

Statistical analysis

Data are expressed as a mean \pm standard error (SEM) calculated over three independent experiments performed in triplicate. Analysis of statistical significance were performed by ANOVA with Bonferroni post hoc test (adhesion and cytotoxicity assays) or Student's *t*-test (IL-8 secretion, NF- κ B and AP-1 activation assays)

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Authors' contributions

AM carried out most experiments and analyzed most of the data. NC wrote the manuscript, participated in the design of the study and analyzed most of the data. MG carried out the IL-8 ELISA assay. OL carried out the construction of NF- κ B reporter cells. KR carried out the construction of AP-1 reporter cells. JD and HB participated in the design of the construction of NF- κ B and AP-1 reporter cells and help to draft the manuscript. PS and NO were involved in the design of the study. MF participated in the design of the study and writing of the manuscript, AG performed the statistical analysis. All authors read and approved the final manuscript.

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Pseudomonas fluorescens alters epithelial permeability and translocates across Caco-2/TC7 intestinal cells

Amar Madi¹, Pascal Svinareff², Nicole Orange¹, Marc GJ Feuilleley¹, Nathalie Connil^{1*}



RESEARCH

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Pseudomonas fluorescens alters epithelial permeability and translocates across Caco-2/TC7 intestinal cells

Amar Madi¹, Pascal Svinareff², Nicole Orange¹, Marc GJ Feuilloley¹, Nathalie Connil^{1*}

Abstract

Background: *Pseudomonas fluorescens* has long been considered as a psychrotrophic microorganism. Recently, we have shown that clinical strains of *P. fluorescens* (biovar 1) are able to adapt at a growth temperature of 37°C or above and induce a specific inflammatory response. Interestingly, a highly specific antigen of *P. fluorescens*, I2, is detected in the serum of patients with Crohn's disease but the possible role of this bacterium in the disease has not yet been explored. In the present study, we examined the ability of a psychrotrophic and a clinical strain of *P. fluorescens* to modulate the permeability of a Caco-2/TC7 intestinal epithelial model, reorganize the actin cytoskeleton, invade the target cells and translocate across the epithelium. The behaviour of these two strains was compared to that of the well known opportunistic pathogen *P. aeruginosa* PAO1.

Results: Both strains of *P. fluorescens* were found to decrease the transepithelial resistance (TER) of Caco-2/TC7 differentiated monolayers. This was associated with an increase in paracellular permeability and F-actin microfilaments rearrangements. Moreover, the invasion and translocation tests demonstrated that the two strains used in this study can invade and translocate across the differentiated Caco-2/TC7 cell monolayers.

Conclusions: The present work shows for the first time, that *P. fluorescens* is able to alter the intestinal epithelial barrier function by disorganizing the F-actin microfilament network. Moreover, we reveal that independently of their origins, the two *P. fluorescens* strains can translocate across differentiated Caco-2/TC7 cell monolayers by using the transcellular pathway. These findings could, at least in part, explain the presence of the *P. fluorescens* specific I2 antigen in the serum of patients with Crohn's disease.

Background

Pseudomonas fluorescens has long been considered as a psychrotrophic microorganism, unable to grow at temperatures above 32°C. Recently, we have shown that clinical strains of *P. fluorescens* (biovar 1) have been able to adapt at a growth temperature of 37°C or above [1]. This event appears relatively frequently and can lead to strains of increased virulence, in the range of the well known opportunistic pathogen *Pseudomonas aeruginosa*. Some of these clinical strains of *P. fluorescens* have a high hemolytic activity [2] presumably associated with type III or VI secretion systems and were shown to induce cytotoxic and proinflammatory responses on epithelial

intestinal cells [3]. These observations and the incidence of declared nosocomial infections due to this germ [4] have changed the vision of the potential risk associated with *P. fluorescens*. This gram-negative bacterium is widespread in the environment where it is even more frequent than *P. aeruginosa* and has a remarkable fitness potential [5]. *P. fluorescens* is found in many ecological niches including soil and water but also in refrigerated food products where its psychrotrophic character gives it the possibility to grow in the relative absence of competitors [6].

P. fluorescens can also be found as a low level commensal of the human digestive tract [7]. Interestingly, a highly specific antigen of *P. fluorescens*, designated as I2, was detected in the serum of 54% of the patients suffering from ileal Crohn's disease (CD) [8] and a correlation between the severity of the pathology and the level of the circulating I2 antigen has been demonstrated [9].

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A relation between exposure to psychrotrophic germs and CD is suggested by the parallel emergence and rise of the pathology in western countries and the widespread use of refrigerated food [10]. Although CD, as other chronic inflammatory bowel diseases, is typically a multifactorial pathology, these data underline the interest of better understanding of the possible interactions between *P. fluorescens* and the intestinal epithelium.

Bacterial pathogens can cause exacerbations or relapse of ulcerative colitis and CD. A healthy intestinal epithelium acts as a barrier between foreign antigens and the underlying lymphoid tissue. Many pathogens have developed strategies to circumvent the barrier function of an intact epithelium. These include inflammatory induction, physical disruption of the barrier and crossing the epithelial cell monolayer. The barrier function is maintained by tight junctions, which are dynamic cell-cell adhesions that form a continuous seal around the cells and control the permeability of solutes and fluids diffusing through the paracellular pathway [11]. Tight junctions are formed by transmembrane proteins, such as claudins and occludin, and cytosolic proteins recruited to the apicolateral membrane, including zonula occludens proteins, cingulin and 7H6 [12,13]. Cytoskeletal components are anchored to tight junction structures through the cytosolic proteins, forming a perijunctional acto-myosin ring which gives the junction architectural support. These components of the cytoskeleton may also be involved in shuttling signalling molecules to and from the junctions [14,15]. Consequently, tight junctions can be disrupted by rearrangement and contraction of the perijunctional acto-myosin ring and this could lead to variations in transepithelial resistance (TER). Alteration of the epithelial barrier function by pathogens can be associated with translocation of the microorganisms across the monolayer. Such mechanisms are used by many enteropathogens, like *Salmonella*, *Yersinia*, *Shigella* or *Listeria*, to disseminate within the host. Three translocation mechanisms are relevant to the intestinal epithelium: (i) exploitation of M cells, specialized intestinal epithelial cells which transport luminal antigens across the epithelial barrier to underlying lymphoid tissue; [16] transcellular translocation or transcytosis, in which pathogens invade epithelial cells, migrate across the cytoplasm and egress from the opposite surface; and (iii) paracellular translocation or paracytosis, in which bacteria travel extracellularly through tight junctions between adjacent epithelial cells. Translocation across intestinal epithelial cells barriers via all these three mechanisms has been reported for *Campylobacter jejuni* which in turn promotes the translocation of other commensal bacteria [17,18].

We have recently shown that *P. fluorescens* can adhere to intestinal epithelial cells, has cytotoxic activity and induces a specific inflammatory response [3], but the ability of this bacterium to alter the intestinal epithelial

barrier function or to translocate across this epithelium has not yet been studied until now. In the present study, we examined the ability of a psychrotrophic and a clinical strain of *P. fluorescens* to modulate the permeability of a Caco-2/TC7 intestinal epithelial model, reorganize the actin cytoskeleton, translocate across the epithelium and invade the target cells. The behaviour of these two strains was compared to that of the well known opportunistic pathogen *P. aeruginosa* PAO1.

Methods

Bacterial strains

P. fluorescens MFN1032 (biovar I) was collected in a hospital of Haute-Normandie (France). It was characterized by polyphasic identification, 16 S RNA sequencing and siderotyping [1]. *P. fluorescens* MF37 is a spontaneous rifampicin-resistant mutant of the strain MF0 (biovar V), originally identified in crude milk [19]. *P. aeruginosa* PAO1 was obtained from an international collection. All the strains were cultivated in ordinary nutrient broth (Merk, Darmstadt, Germany), at 28°C for the two strains of *P. fluorescens* or 37°C for *P. aeruginosa* PAO1.

Cell line and culture

The Caco-2/TC7 clone established from the parental human enterocytes-like Caco-2 cell line [20] was used between passages 25 and 35. Cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM, invitrogen) supplemented with 15% heat-inactivated fetal calf serum, 2 mM of L-glutamine, 100 U/ml each of penicillin and streptomycin and 1% non essential amino acids. For experimental assays, the cells were seeded at a density of approximately 10^5 cells/cm² in 24-well tissue culture plates or on inserts (6.4 mm diameter, 3 µm pore size, Falcon) which allow epithelial differentiation between apical and basolateral compartments. The cells were cultured at 37°C in 5% CO₂-95% air atmosphere and the medium was changed daily. Caco-2/TC7 cells grown in 24-well tissue culture plates were incubated to early confluence (undifferentiated cells). Caco-2/TC7 cells grown on inserts were used at 21 days post-confluence (fully differentiated cells).

Cell infection

Bacteria in early stationary phase (grown overnight) were harvested by centrifugation (5000 g, 5 min, 20°C) and resuspended at a density of 10^8 colony forming unit (CFU)/ml in cell culture medium without serum and antibiotics. Caco-2/TC7 grown on 24-well culture plates (undifferentiated cells) or inserts (fully differentiated cells) were washed twice with fresh cell culture medium and the bacterial suspensions were applied to the surface or to the apical compartments at a concentration of 10^8 CFU/cm². Infected cells were then incubated at

37°C in 5% CO₂-95% air during 24 h for permeability, 3 or 6 h for translocation and 4 h for invasion assays. Each assay was conducted in triplicate in independent experiments (successive passages of Caco-2/TC7 cells).

Transepithelial resistance measurements

The transepithelial resistance (TER) of differentiated Caco-2/TC7 monolayers was studied during 24 h, using the Millicell Electrical Resistance System (Millipore Corp, Bedford, MA). TER values are expressed as percentages of the pre-infection level of the TER (baseline) measured for each individual cell monolayer in the inserts.

Cytotoxicity assay

At the end of infection, the supernatants from Caco-2/TC7 monolayers were collected and the concentration of lactate dehydrogenase (LDH), a cytoplasmic enzyme released upon cell death, was determined using an enzymatic assay (Cytotox 96 Promega, Charbonnières, France) as previously described [3].

Quantification of paracellular flux

Paracellular permeability was quantified using the fluorescein isothiocyanate dextran tracer 4 kDa (FD-4). One hour before the end of infection, 1 mg/ml of FD-4 was added to the apical side of differentiated Caco-2/TC7 monolayers and the amount of FD-4 translocated to the basolateral medium was then measured by fluorimetry using a TD700 fluorometer (Turner Designs) (485 nm excitation and 528 nm emission wavelengths). The results are expressed as a percentage of apical dextran that crossed the insert membrane per cm² and per hour as described by Lynch *et al* [21].

Actin visualisation

At the end of infection, apical F-actin cytoskeleton was visualized. Briefly, differentiated Caco-2/TC7 monolayers were washed with phosphate-buffered saline (PBS), fixed for 10 min in 3.7% paraformaldehyde and permeabilized for 5 min with 0.1% Triton X100 at room temperature. The cells were incubated with 1% bovine serum albumine in PBS for 10 min. F-actin was stained by incubation with Alexa-488 phalloïdine (1 U/insert) for 45 min at room temperature. Following three washes in PBS, cell monolayers were excised from the filter supports, mounted on slides using Fluoromount/Plus mounting medium (Diagnostic BioSystems) and examined using a confocal laser scanning microscope (Zeiss, LSM710).

Bacterial translocation

After 3 and 6 h of bacterial infection, aliquots of 100 µl of the basolateral compartment were collected and the number of bacteria that crossed the epithelial monolayers was determined by serial dilution and plating

onto nutrient agar. *P. fluorescens* and *P. aeruginosa* are aerobic bacteria which require free access to oxygen for a rapid multiplication. Indeed, control studies showed that in our experimental conditions, the growth of *P. fluorescens* and *P. aeruginosa* in the basolateral compartment of the inserts was negligible. This observation allows to consider that the bacterial population measured in the basolateral compartment is actually corresponding to bacteria that crossed the epithelial barrier.

Bacterial invasion assay

After 4 h of infection, Caco-2/TC7 monolayers grown in 24-well culture plates or inserts were washed with PBS. Adherent bacteria were killed by incubation for 1 h with 300 µg/ml gentamycin, an antibiotic that does not cross the cytoplasmic membrane of eukaryotic cells and then only kills bacteria not internalized in cells. Caco-2/TC7 monolayers were washed 3 times with PBS to remove the antibiotic and dead bacteria and were lysed by incubation for 15 min with 0.1% Triton X100 to release the intracellular bacteria. The lysates were then plated onto nutrient agar to determine the number of internalized bacteria.

Statistical analysis

All experiments were conducted independently at least three times. The results are expressed as means ± standard error of the mean [22] and statistical significance were performed by ANOVA with Bonferroni post hoc test.

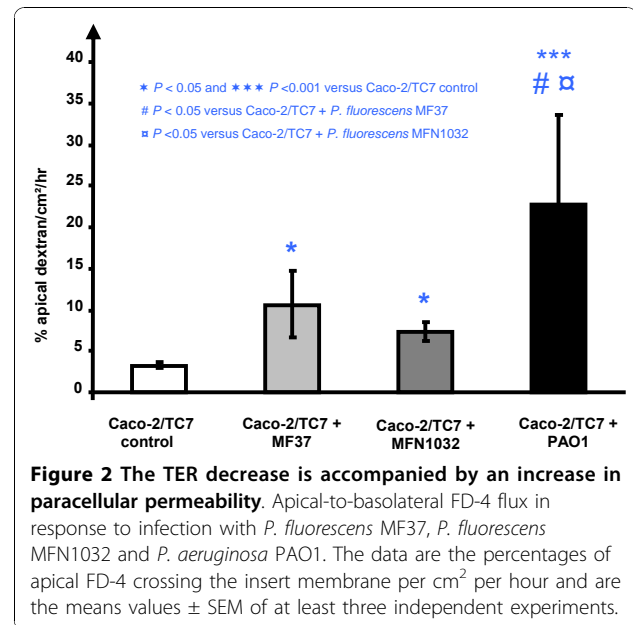
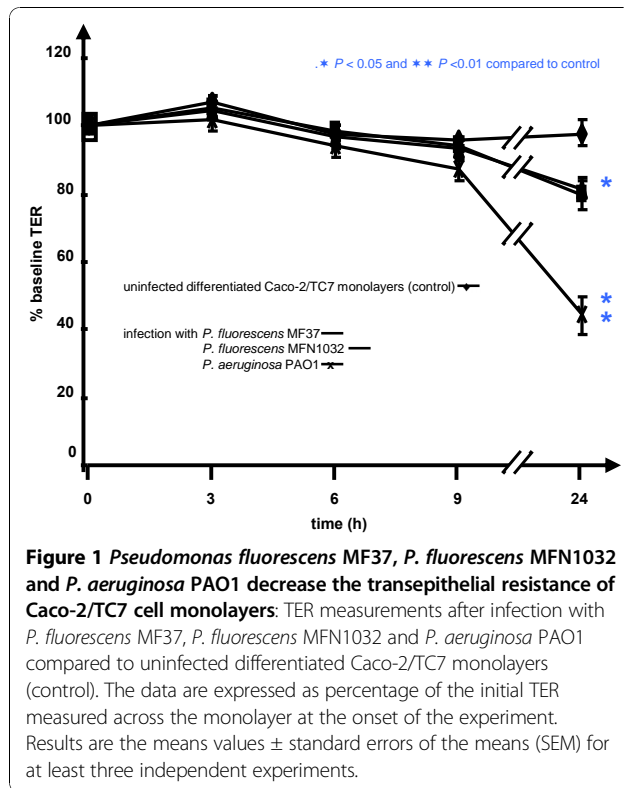
Results

P. fluorescens can alter the epithelial barrier function

The effect of *P. fluorescens* MF37, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 infection on epithelial permeability was evaluated by measuring the TER across differentiated Caco-2/TC7 monolayers (Figure 1). TER values were measured at the onset of the experiment and at times 3, 6, 9 and 24 h. Up to 9 h after the beginning of the experiment, the TER values of the monolayers exposed to *P. fluorescens* MF37, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 remained unchanged. After 24 h of infection, the TER of the monolayers exposed to the two strains of *P. fluorescens* were significantly decreased ($-20.3 \pm 3.9\%$ for MF37 and $-18.3 \pm 3.1\%$ for MFN1032). *P. aeruginosa* led to a deeper decrease of the TER value ($55.8 \pm 5.3\%$). The fall in TER can not be attributed to damages provoked by acidification of the medium since the pH of the medium remained constant over the studies.

The decrease of TER is associated with an increase in paracellular permeability

The observed decrease in TER can have three causes: (i) an increase of paracellular permeability, [16] cell lysis



and epithelium disorganization or (iii) variations of ion fluxes across an intact monolayer. To distinguish between these possibilities, cell lysis was estimated by an assay of LDH released upon cell death after 24 h of infection and the paracellular permeability was quantified by measuring the apical to basolateral flux using 4 kDa dextran tracer (FD-4). Only *P. aeruginosa* PAO1 had a marginal cytotoxic effect on differentiated Caco-2/TC7 cell monolayers after 24 h of incubation (data not shown). In contrast, the three tested bacteria provoked a significant increase in the paracellular permeability after 24 h of incubation (Figure 2). The paracellular permeability of control differentiated Caco-2/TC7 monolayers was low ($3.2 \pm 0.3\%$ of apical FD4 crossing the epithelial barrier/ cm^2/h). *P. aeruginosa* PAO1 caused the greatest increase of paracellular permeability with $22.8 \pm 10.8\%$ of apical FD-4 crossing the barrier/ cm^2/h . The effect of *P. fluorescens* MF37 and *P. fluorescens* MFN1032 was less pronounced but reached $10.6 \pm 4.1\%$ and $7.4 \pm 1.2\%$ FD-4 crossing/ cm^2/h , respectively.

P. fluorescens induces apical F-actin filaments reorganization

Because F-actin filaments in the apical poles of epithelial cells are intimately linked to and regulate tight junction functions, we looked at the effect of *P. fluorescens* MF37, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 on the

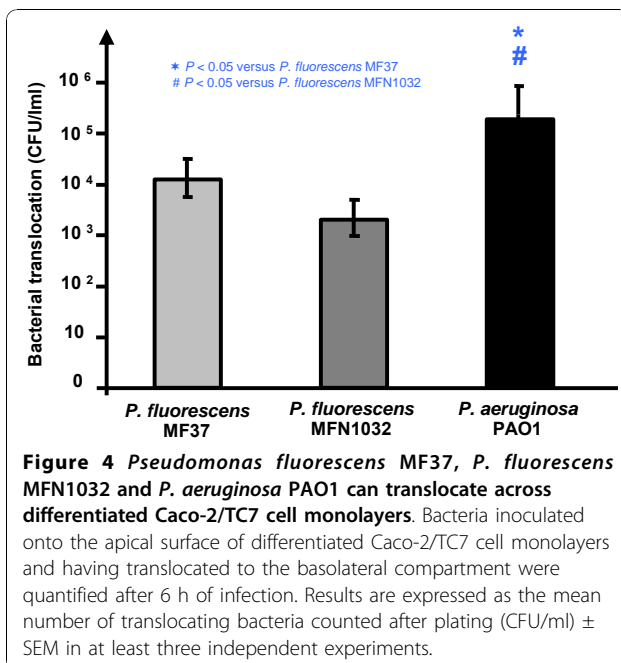
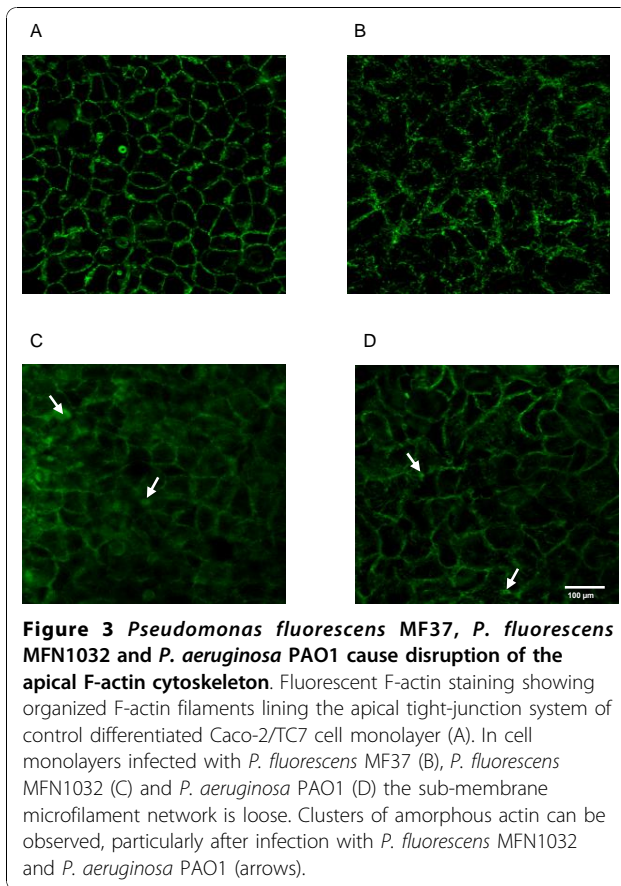
organization of the sub-membrane microfilament network. As shown in Figure 3, incubation with bacteria induced a dramatic reorganization of apical epithelial F-actin. The staining pattern of untreated Caco-2/TC7 showed a continuous fine meshwork of microfilaments lining the cell border. In cells exposed for 24 h to the three bacterial strains, the normal organization of microfilaments was replaced by a diffused and loose network of F-actin fibers. Moreover, these changes were accompanied by the formation of dense clusters of apparently amorphous F-actin, particularly after treatment with the clinical strain *P. fluorescens* MFN1032 and with *P. aeruginosa* PAO1.

P. fluorescens translocates across Caco-2/TC7 monolayers

After apical infection of differentiated Caco-2/TC7 monolayers, bacterial strains were quantified in the basolateral medium at different times. After three hours of infection, bacteria were not detectable in the basolateral compartment. However, after 6 h of infection, $1.5 \pm 2.2 \times 10^4$, $2.9 \pm 2.5 \times 10^3$ and $7.2 \pm 2.2 \times 10^5$ CFU/ml were detected with *P. fluorescens* MF37, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1, respectively (Figure 4). These values of bacterial translocation remain marginal compared to the size of the initial inoculum (10^8 CFU). Nevertheless, if we consider that these results indicate that 1/1000 to 1/10,000 of *Pseudomonas* can cross the epithelium within 6 h, these values may be highly significant in a situation of chronic exposure to these bacteria.

P. fluorescens can translocate without disrupting Caco-2/TC7 cell monolayer integrity

Bacterial translocation through the monolayer can result either from trans- or paracellular passage of the bacteria



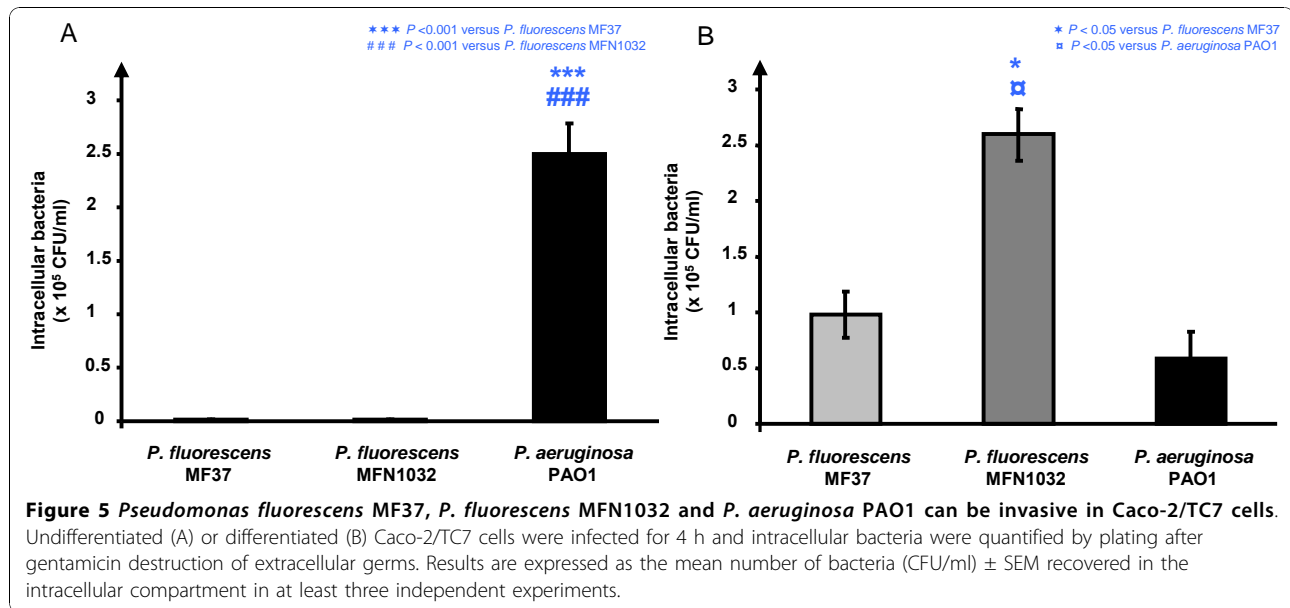
or more simply from gaps formed in the monolayer following the death of some epithelial cells. The TER was evaluated at different times and, as previously shown in Figure 1, this value remained identical for all tested bacteria between the onset of the experiment and 9 h of incubation. This absence of variation of TER indicates that the monolayer integrity was maintained at least 9 h after the infection. These results are in agreement with measures taken using FD-4 between 0 and 9 h of incubation with bacteria that showed no significant variations of paracellular permeability during these short incubation periods (data not shown).

P. fluorescens can be invasive in Caco-2/TC7 cells

Since the differentiated Caco-2/TC7 monolayer remained intact and the paracellular permeability was unchanged at times when bacteria were actually recovered in the basolateral compartment, these results suggested that *Pseudomonas* are able to invade epithelial cells, migrate across the cytoplasm and egress at the level of the basal membrane. This possible transcellular translocation of *Pseudomonas* was investigated using the gentamicin exclusion test. The behaviour of bacteria was different in undifferentiated and differentiated cells. In undifferentiated Caco-2/TC7 cells, only *P. aeruginosa* PAO1 was invasive (2.5×10^5 CFU/ml) whereas no *P. fluorescens* MF37, or *P. fluorescens* MFN1032 were detected in the intracellular compartment (Figure 5A). The situation was different in differentiated Caco-2/TC7 monolayers where the three bacterial strains had clearly an invasive behaviour with $0.9 \pm 0.2 \times 10^5$, $2.6 \pm 0.2 \times 10^5$ and $0.6 \pm 0.3 \times 10^5$ CFU/ml detected in the intracellular compartment for *P. fluorescens* MF37, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1, respectively (Figure 5B). It is interesting to note that in the differentiated monolayer, the two strains of *P. fluorescens* were more invasive than *P. aeruginosa* PAO1, and the level of the clinical strain of *P. fluorescens* MFN1032 was more than 4 times that of the *P. aeruginosa* strain

Discussion

Precise regulation of intestinal epithelial tight junctions is crucial to maintain the barrier function between the luminal compartment and the interior medium. Numerous mucosal pathogens can modulate tight junctions and translocate across the epithelial barrier leading to established disease states. In this regard, our pioneer works [23,24] and the recent studies demonstrating that *P. fluorescens* can clearly act as an opportunistic pathogen [3,25] underscore the need to further characterize the virulence potential of this microorganism. In the present work, we studied the translocation and invasion potential of *P. fluorescens* on differentiated Caco-2/TC7 epithelial intestinal cell monolayers and the effect of the



bacteria on paracellular permeability and tight junctions associated actin cytoskeleton. The behaviour of these bacteria was compared to that of the opportunistic pathogen *P. aeruginosa* PAO1.

We demonstrate that, independently of their maximal growth temperature, the two strains of *P. fluorescens* MF37 and MFN1032, respectively from environmental and clinical origin, can lead to a time-dependent decrease of the epithelial barrier function. This effect starts after 9 h of contact between the bacteria and the epithelial cells but is more pronounced at 24 h post-infection. This decrease of TER is lower than that induced by *P. aeruginosa* PAO1. This result is logical since it has been demonstrated that the *P. aeruginosa* quorum sensing factor N-(3 oxodecanoyl)-L-homoserine lactone can disrupt the epithelial barrier integrity of Caco-2 cells [26] whereas *P. fluorescens* is not known to produce N-acyl homoserine lactones. The quorum sensing factors expressed by this species remain to be identified. Compared to some other bacteria such as *Escherichia coli* O157 which are known to cause a rapid decrease in TER [27], the decrease of TER observed after treatment with the two *P. fluorescens* strains presently studied was slow, suggesting that this effect on TER is mediated by synthesis of active molecules either by epithelial cells and/or bacteria. Indeed, *P. fluorescens*, and particularly a strain such as MFN1032, is known to produce exoenzymes but also cyclo-lipopeptides [28]. On the other hand, it is now obvious that many eukaryotic communication factors, including peptides locally produced by the intestinal epithelium, can enhance the virulence of *Pseudomonas* [29]. A decrease of TER can result either from an increase in paracellular permeability, local cell lysis in the

monolayer or change in ion flux across the intact monolayer. To distinguish between these possibilities, LDH released by lysed cells was quantified and fluorescent markers of paracellular flux were used. We have previously shown that *P. fluorescens* MF37 and *P. fluorescens* MFN1032 can exert a cytotoxic effect on confluent undifferentiated epithelial intestinal cells [3]. Surprisingly, on fully differentiated Caco-2/TC7 cells cultivated on insert, no significant cytotoxicity was observed, even after 24 h of infection with these two strains. This indicates that the increase of TER is not due to direct toxic effects and cell lysis. Only infection with *P. aeruginosa* PAO1 provoked a modest lysis of fully differentiated Caco-2/TC7 cells explaining the higher effect of this strain on the TER values. It has been shown that, as the epithelial barrier differentiates and becomes highly polarized, it is more resistant to *P. aeruginosa* infection [30]. This could explain the rather low cytotoxicity of the three *Pseudomonas* species or strains presently studied in comparison with our previous results obtained on undifferentiated cells [3].

The integrity of the epithelial cell monolayer is maintained by intercellular junctional complexes composed of tight junctions, adherens junctions and desmosomes. The tight junctions are the most apical intercellular junctions. They form a continuous belt-like structure at the luminal end of intercellular space that regulates the paracellular flux [31]. Some bacterial pathogens can manipulate the apical-junctional complex and one of the principal strategies used by these microorganisms is to trigger actin cytoskeleton contraction [32]. In the present study, the confocal laser scanning microscope examination of apical F-actin revealed that *P. fluorescens*

MF37, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 induced a profound reorganization of the enterocyte actin cytoskeleton. This effect is very similar to that reported in cells exposed to *Vibrio parahaemolyticus* [21] or enteropathogenic *E. coli* [33]. These observations are in agreement with the results obtained by measurement of TER and FD-4 flux across the cell monolayer.

Our recent observations indicate that *P. fluorescens* induces the secretion of the proinflammatory cytokine interleukin (IL)-8 in epithelial intestinal cells [3]. IL-6, IL-1 β and TNF- α secretion are also induced in human cultured A549 pulmonary cells by *P. fluorescens* [25]. Proinflammatory cytokines released in the gut can increase paracellular permeability by modulating tight junction protein expression and phosphorylation [34]. Moreover, modest inflammatory and metabolic stress are known to promote the translocation and transcytosis of commensal bacteria such as *E. coli* C25 across gut enterocytes [35]. Consequently, we investigated the ability of *P. fluorescens* MF37, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 to translocate across the Caco-2/TC7 cell monolayers. We found that although the translocation is limited compared to the initial inoculum, these bacteria were observed to readily translocate across Caco-2/TC7 monolayers after 6 h of infection. Most importantly, TER measurements and FD-4 flux indicate that at this time, the cell monolayer integrity was maintained in the presence of the translocating bacteria. Numerous mucosal bacteria, such as *Salmonella typhimurium*, translocate across the epithelial barrier and this translocation is associated with a complete loss of TER after 6 h [36]. Pathogen bacteria of the *Pseudomonas* genus are generally considered as extracellular microorganisms. However, these bacteria have been shown to have an invasive behaviour in specific eukaryotic cells such as corneal epithelial cells for *P. aeruginosa* [37] and cortical neurons for *P. fluorescens* [38]. Our results suggest that the translocation of *Pseudomonas*, through the cell monolayers, occurs after invasion of the differentiated cells. It is known that *S. typhimurium* and *E. coli* M12 are more invasive in immature undifferentiated than in differentiated Caco-2 cells [39]. In contrast, in our study the gentamicin exclusion test demonstrates that while *P. aeruginosa* PAO1 invade both polarized and non-polarized Caco-2/TC7 cells, *P. fluorescens* only invades the differentiated cells. As expected for a clinical strain, *P. fluorescens* MFN1032 had a higher invasive activity than its psychrotrophic and environmental counterpart *P. fluorescens* MF37. However, despite this higher invasion potential, the translocation level of *P. fluorescens* MFN1032 was similar to that of MF37. These observations can be explained by the fact that polarized epithelial cells are characterized by the presence of membrane proteins

distributed to apical or basolateral surfaces, and these proteins may be absent or poorly expressed in undifferentiated epithelial cells. Then, bacteria may preferentially interact with these proteins, exhibiting affinity for either apical or basolateral surfaces, or for differentiated or undifferentiated epithelial cells [40].

Conclusion

Our results reveal that the psychrotrophic strain *P. fluorescens* MF37 and the clinical strain *P. fluorescens* MFN1032 have very similar effects on Caco-2/TC7 cells and can contribute to a decrease in the barrier effect of the intestinal epithelium. As these bacteria have a low cytotoxic activity, in these cells, they apparently disorganize intercellular junctions by acting on the F-actin microfilament network. Moreover, we have shown that independently of its origins and physiological variations, *P. fluorescens* can translocate across differentiated Caco-2/TC7 cell monolayers by using the transcellular pathway. These findings could, at least in part, explain the presence of the *P. fluorescens* specific I2 antigen in the serum of patient with CD. Further studies of the effects of this bacterium on the expression and distribution of tight junction proteins of intestinal epithelial cells, may help to understand the molecular mechanism used by this bacterium to modulate the intestinal epithelial barrier function.

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Authors' contributions

AM carried out most experiments and analyzed most of the data. NC wrote the manuscript and participated in the design of the study. PS and NO helped to draft the manuscript. MF participated in the design of the study and writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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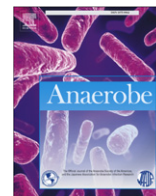
In vitro evaluation of the probiotic potential of *Lactobacillus salivarius* SMXD51

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ABSTRACT

Lactobacillus salivarius SMXD51 was previously isolated from the cecum of a Tunisian poultry and found to produce a bacteriocin-like substance highly active against the foodborne pathogen *Campylobacter jejuni*. The aim of this study was to examine some probiotic properties of the strain: acid and bile tolerance, capacity of adhesion, stimulation of immune defences (IL-6, IL-8, IL-10 and β -defensin 2), and modulation of the barrier integrity. The results showed that *L. salivarius* SMXD51 can tolerate gastrointestinal conditions (acid and bile), adhere to intestinal cells and stimulate the immune system. The bacterium strengthened the intestinal barrier functions through the increase of the transepithelial electrical resistance (TEER) and reinforcement of the F-actin cytoskeleton. One hour pretreatment with *L. salivarius* SMXD51 protected against *Pseudomonas aeruginosa* PAO1-induced decrease of TEER and damage of the F-actin cytoskeleton. Our results highlight that *L. salivarius* SMXD51 fulfils the principle requirements of an efficient probiotic and may be seen as a reliable candidate for further validation studies in chicken.

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1. Introduction

Antibiotics have been extensively used in animal feed to improve production in poultry and piglet industries [1]. However, the use of these substances as growth promoters can lead to the development of antibiotic resistances. Such resistances can occur not only in pathogenic bacteria [2,3] which can be transferred from poultry products to human population [4], but also in commensal bacteria [5], constituting a reservoir of resistance genes for pathogenic bacteria [6]. Due to their potential to reduce enteric disease in poultry, probiotics are considered to be a good alternative to the use of antibiotics [7].

It is well-known that probiotics have a number of beneficial health effects in humans and animals. They play an important role in the protection of the host against harmful microorganisms and also strengthen the immune system [8]. Some probiotics have also been found to improve feed digestibility and reduce metabolic disorders [9]. They must be safe, acid and bile tolerant, able to

adhere and colonize the intestinal tract [8]. Human enterocyte-like Caco-2 cells have often been used for *in vitro* studies on the mechanisms of cellular adhesion of nonpathogenic *Lactobacilli* [10,11]. Some probiotic strains were shown to prevent attachment and invasion of various bacterial pathogens. *Lactobacillus* and *Bifidobacterium* were found to counteract *Listeria* infection of cultured epithelial cells through both secretion of antagonist molecules and modulation of the epithelial cell's immune response [12]. Compounds secreted by *Lactobacillus* were also shown to decrease *in vivo* the intestinal colonization by pathogenic strains of *Escherichia coli* [13]. Another and synergistic mechanism by which probiotics exert their beneficial effects is the enhancement of the intestinal barrier function. It has been found that treatment with probiotic bacteria may prevent or reverse increased permeability of the epithelium induced by pathogens [14]. These results were essentially obtained by measurement of the transepithelial electrical resistance (TEER) which appears as a reliable method to evaluate *in vitro* the epithelial permeability and compare the probiotic activity of bacterial species [15].

The genus *Lactobacillus* is commonly found in abundance in the upper gastrointestinal tract of humans and animals. Among this genus, within the recent years, *Lactobacillus salivarius* has gained attention as a promising probiotic species [16,17] that influences cytokine profiles and modulates cellular responses to pathogenic challenge. In a recent study, we isolated *L. salivarius* SMXD51 from

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the cecum of a Tunisian poultry [18]. This strain was found to produce a bacteriocin-like substance active against *Campylobacter jejuni*, a bacterium known as a foodborne pathogen in humans and a common commensal of poultry [19]. The bacteriocin, salivaricin SMXD51 was purified and characterized [20]. In addition to *Campylobacter*, salivaricin SMXD51 showed inhibition against a number of foodborne pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella*. According to these results, *L. salivarius* SMXD51 may be a promising bioprotective strain for the control of pathogenic bacteria in meat products so in order to get more information about this interesting strain, we recently sequenced its genome [21].

In the present work, we aimed to investigate the *in vitro* probiotic potential of *L. salivarius* SMXD51 to evaluate if this strain could be considered as good alternative to antibiotics use in poultry production.

2. Materials and methods

2.1. Bacterial strains

L. salivarius SMXD51, isolated from the cecum of a Tunisian poultry [18] was cultivated at 37 °C in De Man-Rogosa-Sharpe (MRS) medium [22] for 18–24 h. *Pseudomonas aeruginosa* PAO1, a laboratory reference strain, was cultivated at 37 °C in ordinary nutrient broth (Merck, France).

2.2. Acid and bile tolerance

Acid and bile tolerance were studied according to the protocol used by Anderson et al. (2010) [23]. Briefly, *L. salivarius* SMXD51 was grown overnight at 37 °C in MRS broth and suspended to an approximate cell concentration of 10⁸ CFU/ml in MRS broth adjusted to pH 3 for 2 h and in 0.5% w/v bile (Sigma–Aldrich, France) for 4 h. These conditions were chosen to represent the time it takes to pass through the gastrointestinal system and the pH value and bile concentration respectively found in the chicken stomach and intestine. Bacterial viability was assessed by enumeration on MRS agar plates.

2.3. Cell line and culture

The Caco-2/TC7 cells were used between passages 35 and 50. Cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, France) supplemented with 15% heat-inactivated foetal calf serum, 2 mM of L-glutamine, 100 U/ml each of penicillin and streptomycin and 1% non-essential amino acids. For experimental assays, the cells were seeded at a density of approximately 10⁵ cells/cm² in 24-well tissue culture plates (for adhesion, cytotoxicity assay, cytokines and β -defensin 2 quantification) or on inserts (6.4 mm diameter, 3 μ m pore size, Falcon) which allow epithelial differentiation (for TEER assay and actin visualisation). The cells were cultured at 37 °C in 5% CO₂-95% air atmosphere and the medium was regularly changed. Caco-2/TC7 grown in 24-well tissue culture plates were incubated to early confluence (undifferentiated state) whereas Caco-2/TC7 cells grown on inserts were used at 21 days post-confluence (fully differentiated state).

2.4. Adhesion capacity

L. salivarius SMXD51 was harvested by centrifugation, resuspended in cell culture medium without serum and antibiotics at a concentration of 10⁸ CFU/ml, and then applied on confluent Caco-2/TC7 monolayers. After 4 h of incubation at 37 °C, 5% CO₂,

monolayers were washed with PBS to remove non adherent bacteria, and lysed by incubation for 15 min with 0.1% Triton X100. The lysates were then diluted and plated onto MRS agar to determine the number of adherent bacteria.

2.5. Cytotoxicity assay

After 24 h of incubation with *L. salivarius* SMXD51 (10⁸ CFU/ml), the supernatants from Caco-2/TC7 monolayers grown on 24-well tissue culture plates were collected. The lactate dehydrogenase (LDH) present in the supernatants was measured using the Cytotox 96R enzymatic assay (Promega, France). LDH is a stable cytosolic enzyme of eukaryotic cells, indicator of necrotic cell death when released. Caco-2/TC7 cells exposed to Triton \times 100 (0.9%) were used as a control of total release (100% LDH release) as recommended by the manufacturer's instructions. The background level (0% LDH release) was determined with serum free culture medium. To complete the cytotoxicity assay, the integrity of Caco-2/TC7 monolayers was also estimated by observation with a photonic microscope (\times 400).

2.6. Quantification of IL-6, IL-8, IL-10 and β -defensin 2

After 24 h of treatment with 10⁸ CFU/ml of *L. salivarius* SMXD51, the levels of cytokines (IL-6, IL-8 and IL-10) produced by Caco-2/TC7 cells were measured in the culture supernatant using ELISA Quantikine kits (R&D Systems, France). β -defensin 2 was quantified using the Defensin 2, beta (Human) – ELISA Kit (Phoenix, France).

2.7. Transepithelial electrical resistance measurements

The effect of *L. salivarius* SMXD51 on the transepithelial electrical resistance (TEER) of differentiated intestinal culture was studied during 24 h, using the Millicell Electrical Resistance System (Millipore Corp, Bedford, MA). *L. salivarius* SMXD51 was incubated alone at 10⁸ CFU/ml on Caco-2/TC7 cell monolayers to determine the effect of this bacterium on the epithelial barrier integrity. The differentiated cells were also exposed to *P. aeruginosa* PAO1, a well-known pathogen able to damage the barrier integrity, or pre-incubated with *L. salivarius* SMXD51 for 1 h before infection with the pathogen. For each conditions tested, TEER values were expressed as percentages of the initial level measured in the insert.

2.8. Actin visualisation

Fully differentiated Caco-2/TC7 monolayers were exposed either to *L. salivarius* SMXD51 or *P. aeruginosa* PAO1 for 24 h or pre-incubated for 1 h with *L. salivarius* SMXD51 before infection with the pathogen. At the end of the experiment, the cells were washed with phosphate-buffered saline (PBS), fixed for 10 min in 3.7% paraformaldehyde and permeabilized for 5 min with 0.1% Triton \times 100 at room temperature. The cells were then incubated with 1% bovine serum albumin in PBS for 10 min and the apical F-actin cytoskeleton was stained by incubation with Alexa-488 phalloïdin (Invitrogen, France) for 45 min at room temperature (1U/insert). Following three washes in PBS, cell monolayers were excised from the filter supports, mounted on slides using Fluoromount Plus medium (Clinisciences, France) and examined using a confocal laser scanning microscope (Zeiss, LSM710).

2.9. Statistical analysis

Data are expressed as a mean \pm standard error (SE) calculated over three independent experiments performed in triplicate.

Table 1
Evaluation of the probiotic properties of *Lactobacillus salivarius* SMXD51.

Criteria tested	Results
Resistance to acid	Yes. 99% survival after 2 h at pH 3
Resistance to bile	Yes. 99% survival after 4 h at 0.5% w/v bile
Adherence to intestinal cells	Yes. 10^5 UFC/ml (1% of initial bacteria)
Safety/cytotoxicity	No cytotoxicity towards Caco-2/TC7 (see Fig. 1A) No visible damage on the monolayer (see Fig. 1B)
Modulation of immunity	No-induction of IL-6 (see Fig. 2A) Induction of IL-8, 1.8-fold increase (see Fig. 2B) No-induction of IL-10 (see Fig. 2C) Induction of β -defensin 2, 2.6-fold increase (see Fig. 2D)
Barrier integrity	Enhancement in TEER, 1.2 fold increase (see Fig. 3) Protection of F-actin (see Fig. 4)
Antimicrobial activity against pathogenic bacteria	Yes, previous study (Messaoudi et al., 2012) [16]

Analysis of statistical significance was performed by ANOVA with Student's *t*-test.

3. Results and discussion

L. salivarius SMXD51 was evaluated in regard of the typical probiotic properties proposed by Gupta and Garg (2009) [24] and Neville and O'Toole (2010) [17]. Results are summarized in Table 1.

3.1. Acid and bile tolerance

Resistance to acid and bile is generally considered an essential assessment criterion for probiotic evaluation since the strains have to survive the conditions in the stomach and the small intestine [17]. Thus, the survival of *L. salivarius* SMXD51 was tested for 2 h at pH 3 and 4 h in 0.5% w/v bile. The results show that this strain was able to tolerate acidic conditions and the presence of 0.5% w/v bile without any significant loss of viability (99% survival). In these physiological conditions, the behaviour of *L. salivarius* SMXD51 was similar to the results previously obtained by Kirtzalidou et al. [25] for *L. salivarius* C3 isolated from the infant gut microbiota, and by Yun et al. (2009) [26] for *L. salivarius* LB64 from pig feces.

Sequencing of *L. salivarius* SMXD51 by Kergourlay et al. (2012) [21] revealed that several genes known to be important for acid tolerance

and bile salt resistance were found in the genome of the bacteria, among them an ornithine decarboxylation-antiporter system and a bile salt hydrolase family protein choloylglycine hydrolase. The presence of these genes may partially explain the ability of *L. salivarius* SMXD51 to resist to the gastrointestinal conditions tested.

3.2. Adhesion capacity

Adherence to intestinal epithelial cells is another reliable criterion for the selection of probiotic. *In vitro* test results using Caco-2 cell for the estimation of adhesion capacity to intestinal epithelium cells have been reported to have a good correlation to *in vivo* results [27] and this characteristic is often strain-specific. Adhesion capacity of *L. salivarius* SMXD51 (10^8 CFU/ml) was examined by incubating the bacteria with confluent Caco-2/TC7 monolayers for 4 h. At the end of incubation period, non adherent bacteria were removed by rinsing and the number of adherent germs was determined by plating. In these conditions 10^5 CFU/ml adherent *L. salivarius* SMXD51, corresponding to 1% of the initial population, were recovered in the Caco-2/TC7 monolayers lysates. This strain demonstrates a capacity of adhesion at least identical or higher than *L. salivarius* C3 previously tested by Kirtzalidou et al. (2011) [25]. We found that *L. salivarius* SMXD51 have high hydrophobic affinity towards *n*-hexadecane (data not shown). This characteristic may promote hydrophobic interactions which are generally considered to play an important role in the adherence of microorganisms to eukaryotic cells [28]. Moreover, analysis of the sequence of *L. salivarius* SMXD51 by Kergourlay et al. (2012) [21] showed recently the presence of two genes encoding cell wall-anchored adhesin that may be involved in adherence to eukaryotic cells, a fibronectin binding protein (FbpA) and a potential mucus adhesion-promoting protein (MapA). The *lspA* gene from *L. salivarius* UCC118 reported to mediate adhesion was also found with 92.6% identity.

3.3. Cytotoxicity assay

The safety of lactobacilli and bifidobacteria is well documented. In general, the pathogenic potential of these normal colonizers of the human body is considered quite low [29]. Only very rare cases of human infection (commonly endocarditis) have been reported [30,31]. However, further investigation is warranted for probiotic use in at-risk human populations such as severely immunocompromised subjects, neonates or hospitalized patients. For such sensitive populations, the use of appropriate *in vitro* assays to assess the safety of probiotics should not be precluded. *L. salivarius*

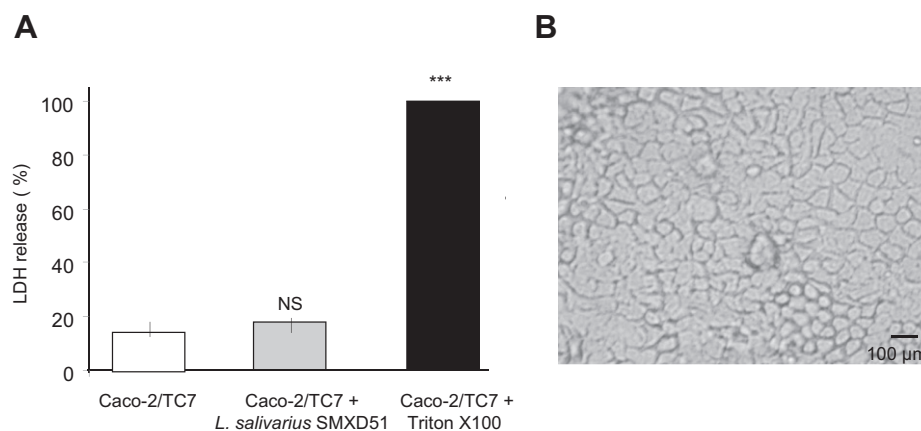


Fig. 1. Cytotoxicity assay. (A) LDH release. Mean \pm SE. NS (not significant) and ****P* < 0.001 compared to Caco-2/TC7 control. (B) Observation of Caco-2/TC7 cells treated with 10^8 CFU/ml of *L. salivarius* SMXD51 with a photonic microscope.

SMXD51 (10^8 CFU/ml) were applied on Caco-2/TC7 monolayers for 24 h and the cytotoxicity was evaluated by measurement of LDH release and microscopic observation. The results show that the amount of LDH release in the supernatant of Caco-2/TC7 cultured in the absence or presence of the bacteria was unchanged, indicating that *L. salivarius* SMXD51 didn't cause cell lysis (Fig. 1A). The absence of cytotoxicity of *L. salivarius* SMXD51 against Caco-2/TC7 cells was confirmed by microscopic studies. Even after 24 h of incubation with the bacteria, the integrity of the monolayer was fully preserved (Fig. 1B).

3.4. Quantification of IL-6, IL-8, IL-10 and β -defensin 2

Another criterion often screened for the selection of probiotics is their capacity to modulate the immune system [32,33]. The quantification of IL-6, IL-8, IL-10 and β -defensin 2 using ELISA assays showed that *L. salivarius* SMXD51 induces 1.8-fold increase in IL-8 secretion (Fig. 2B) and 2.6-fold increase in β -defensin 2 secretion (Fig. 2D) compared to untreated Caco-2/TC7 cells. On the contrary, for IL-6 (Fig. 2A) and IL-10 (Fig. 2C), no variation of the basal secretion of Caco-2/TC7 cells was observed after exposure to the bacteria. These results are consistent with those of Perez-Cano et al. [34] who showed that *Lactobacillus fermentum* CECT5716 and *L. salivarius* CECT5713 display *in vitro* immunomodulatory activity by induction of IL-8. However, in contrast with our results, *L. fermentum* CECT5716 and *L. salivarius* CECT5713 also promoted the production of the pro-inflammatory IL-6 and anti-inflammatory IL-10. These differences are not surprising since the immunomodulatory effects are often strain-specific [35,36]. We also observed that *L. salivarius* SMXD51 induced the secretion of β -defensin 2. It is known that inducible β -defensins play an important role in the intestinal barrier function and *in vitro* studies have shown that clinically effective probiotics induce the production of antimicrobial β -defensin 2 [37–39]. Induction of β -defensin 2 by probiotics, including *L. salivarius* SMXD51, might be an alternative and a complementary new strategy to strengthen innate defence mechanisms.

3.5. Effect of *L. salivarius* SMXD51 on TEER and the F-actin cytoskeleton

Modulation of the barrier integrity was studied to provide further support for the probiotic properties of *L. salivarius* SMXD51.

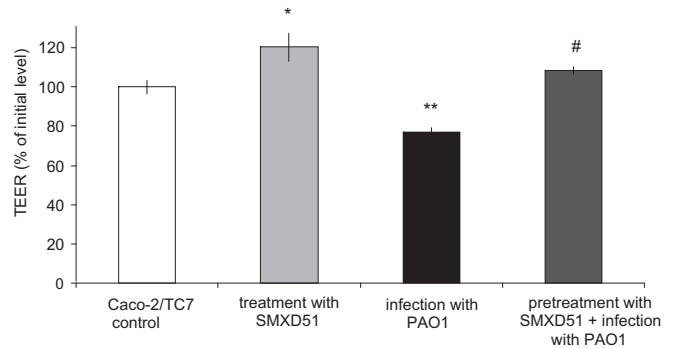


Fig. 3. TEER of Caco-2/TC7 cells exposed to *L. salivarius* SMXD51, *P. aeruginosa* PAO1 or a combination of the two strains. Data are expressed as percentages of the initial level measured in the insert and as a mean \pm SE. * $P < 0.05$ and ** $P < 0.01$ compared to Caco-2/TC7 control. # $P < 0.01$ compared to infection with PAO1.

Caco-2/TC7 cells were treated with *L. salivarius* SMXD51 during 24 h. The results showed that the bacteria induce a $20 \pm 7\%$ increase of the TEER of the monolayer (Table 1, and Fig. 3). Confocal microscopy observations confirmed that the network of the F-actin cytoskeleton was more dense after 24 h incubation with *L. salivarius* SMXD51 (Fig. 4B) compared to an untreated control (Fig. 4A). The use of transepithelial electrical resistance of intestinal epithelial cell monolayers as a mean to evaluate probiotic activity was first proposed by Klingberg et al. [15]. They investigated the probiotic potential of five lactobacilli strains and found that *Lactobacillus plantarum* MF1298 and *L. salivarius* DC5 showed the highest increase in TEER in a dose dependent manner. The maximal increase of TEER obtained was about 40% after 24 h of incubation with the probiotics.

3.6. Protection of the barrier integrity by *L. salivarius* SMXD51

We evaluated if *L. salivarius* SMXD51 can prevent the loss of barrier integrity provoked by a pathogen, *i.e.* *P. aeruginosa* PAO1. This strain was chosen as model for alteration of the intestinal epithelium because it has been previously shown to have a strong cytotoxic activity towards Caco-2/TC7 cells and be able to disrupt the F-actin cytoskeleton [40]. Our results revealed that when Caco-2/TC7 cells were infected with *P. aeruginosa* PAO1 (10^6 CFU/ml), the TEER was decreased by a mean of $25 \pm 3\%$ (Fig. 3) and the F-actin cytoskeleton

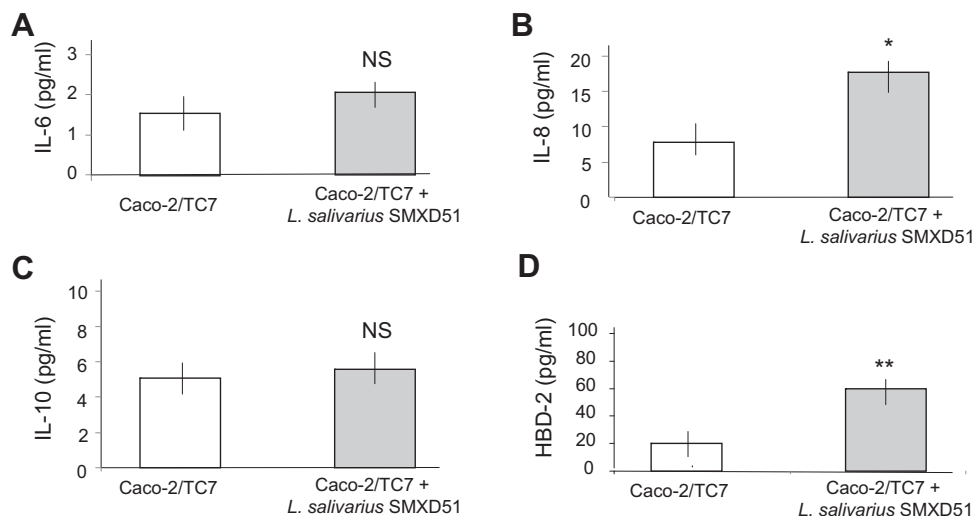


Fig. 2. Quantification of cytokines and β -defensin 2 (HBD-2) in the supernatants of Caco-2/TC7 cells after exposition to *L. salivarius* SMXD51. (A) IL-6, (B) IL-8, (C) IL-10 and (D) HBD-2. Mean \pm SE. NS (not significant), * $P < 0.05$ and ** $P < 0.01$ compared to Caco-2/TC7 control.

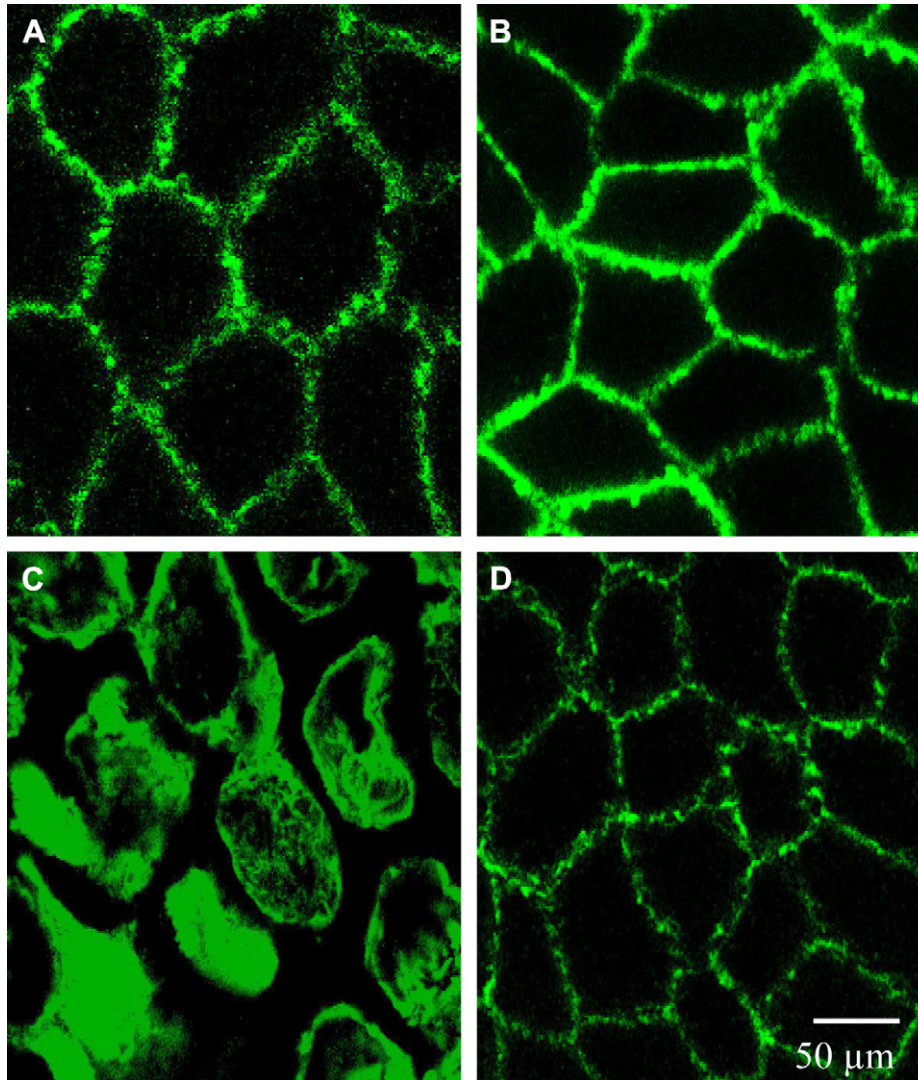


Fig. 4. Staining of F-actin cytoskeleton with Alexa-488 phalloidin of Caco-2/TC7 cells without treatment (A), treated with 10^8 CFU/ml of *L. salivarius* SMXD51 (B), infected with 10^6 CFU/ml of *P. aeruginosa* PAO1 (C), or pretreated with SMXD51 and then infected with PAO1 (D).

was seriously damaged (Fig. 4C). Preincubation of Caco-2/TC7 cells with *L. salivarius* SMXD51 (10^8 CFU/ml) prevented the deleterious *P. aeruginosa* PAO1-induced TEER changes (Fig. 3) and the cytoskeleton disruption (Fig. 4D). However, this effect was only observed when *L. salivarius* SMXD51 is added 1 h before the infection with the pathogen. When the two bacteria were added simultaneously, *L. salivarius* SMXD51 failed to prevent the loss of barrier integrity (data not shown). It could be suggested that when *L. salivarius* SMXD51 is used in prevention to the infection, the bacteria may form a physical protection barrier on the monolayer that refrains the access of the pathogen to the eukaryotic receptor by occupying the target sites. Moreover, during the pretreatment, *L. salivarius* SMXD51 may have time to secrete some molecules that will have antagonistic activities against *P. aeruginosa* PAO1. Similar mechanisms for other probiotics have been described previously. Liu et al. [41] found that *L. plantarum* prevented the decrease in the expression of tight junction proteins and F-actin in NCM460 cells infected with enteroinvasive *E. coli* (EIEC) or enteropathogenic *E. coli* (EPEC). Another study found that *Lactobacillus rhamnosus* GG protected epithelial monolayers against enterohaemorrhagic *E. coli* (EHEC)-induced redistribution of the claudin-1 and ZO-1 tight junction proteins. *Lactobacillus acidophilus* protected against F-actin rearrangement

induced in an epithelial cell line upon exposure to a pathogenic *E. coli* [42]. *Streptococcus thermophilus* and *L. acidophilus* maintained (actin, ZO-1) or enhanced (actinin, occludin) cytoskeletal and tight junctional protein structures in epithelial cell lines [14]. Similarly, *E. coli* Nissle 1917 can counteract the disruptive effects of EPEC on T-84 epithelial cells tight junctions [43].

4. Conclusion

This study shows that *L. salivarius* SMXD51 fulfils to all the principle requirements and properties of an efficient probiotic (tolerance to gastrointestinal conditions, adhesion to intestinal cells, stimulation of immunity, and reinforcement of epithelial barrier). These findings demonstrated that *L. salivarius* SMXD51 could be a reliable candidate for future use as probiotic in chicken feed supplement. Further investigations will be conducted in animal experiments to assess the *in vivo* efficacy of the strain.

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RESEARCH ARTICLE

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Cytotoxicity and inflammatory potential of two *Pseudomonas mosselii* strains isolated from clinical samples of hospitalized patients

Charlène Leneveu-Jenvrin, Amar Madi, Emeline Bouffartigues, Kelly Biaggini, Marc Feuilloley, Sylvie Chevalier and Nathalie Connil*

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Abstract

Background: The genus *Pseudomonas* includes a heterogeneous set of microorganisms that can be isolated from many different niches and nearly 100 different strains have been described. The best characterized bacterium is *Pseudomonas aeruginosa* which is the primary agent of opportunistic infection in humans, causing both acute and chronic infections. Other species like *fluorescens*, *putida* or *mosselii* have been sporadically isolated from hospitalized patients but their association with the pathology often remains unclear.

Results: This study focuses on the cytotoxicity and inflammatory potential of two strains of *Pseudomonas mosselii* (ATCC BAA-99 and MFY161) that were recently isolated from clinical samples of hospitalized patients. The behavior of these bacteria was compared to that of the well-known opportunistic pathogen *P. aeruginosa* PAO1. We found that *P. mosselii* ATCC BAA-99 and MFY161 are cytotoxic towards Caco-2/TC7 cells, have low invasive capacity, induce secretion of human β -defensin 2 (HBD-2), alter the epithelial permeability of differentiated cells and damage the F-actin cytoskeleton.

Conclusions: These data bring new insights into *P. mosselii* virulence, since this bacterium has often been neglected due to its rare occurrence in hospital.

Keywords: *Pseudomonas mosselii*, Cytotoxicity, Inflammation, F-actin cytoskeleton damage

Background

The genus *Pseudomonas* is one of the most diverse and ecologically significant bacterial groups on the planet. The large size and the plasticity of their genome explain at least partly their ability to cope with different forms of stresses (physical, chemical or antimicrobial agents) resulting in their widespread distribution [1]. The genus *Pseudomonas* includes more than 100 species, a number that is increasing in time [2]. Nearly each year, a new species is indeed discovered, like *P. duriflava*, *P. batumici* or *P. litoralis* for example, isolated from a desert soil [3], the Caucasus Black sea coast [4] or from Mediterranean seawater [5], respectively. Due to its heterogeneity, the genus *Pseudomonas* has undergone numerous taxonomic

changes depending on the criteria employed for their definition and delineation: phenotypic, physiologic or metabolic characteristics, siderotyping, phylogeny based on 16S rRNA and/or “housekeeping” genes, analysis of 16S-23S rRNA intergenic spacers (ITS) or the use of functional and ecological genetic markers such as *oprE*, *oprD* or *gacA* [2,6-8].

P. aeruginosa is by far the most studied species in the genus *Pseudomonas*. It is an opportunistic pathogen that provokes nosocomial infection and causes severe acute and chronic infections either in healthy or in immunocompromised individuals [9]. Other *Pseudomonas* species have been suspected in human infections [2]. For example, the very common environmental contaminant *P. fluorescens* has also been associated to various clinical cases [10-14]. This bacterium may particularly colonize the airways, the urinary tract and blood of immunocompromised patients. Recently, some *P. fluorescens* strains

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were found to behave as human pathogens, since they have a high hemolytic activity and dispose of a complete type three secretion system arsenal [15-18].

P. mosselii is a novel species, which has been characterized in 2002 [19]. It has been linked to *P. putida* clinical strains using 16SrDNA, *oprF* and *oprD* as markers for phylogeny-based studies [7,8]. In 2009, McLellan and Partridge [20] presented a case of prosthetic valve endocarditis caused by *P. mosselii*. These authors proposed that *P. mosselii* should be regarded as a potential pathogen.

In a previous study, we have found that *P. mosselii* strains were able to adhere and to display a necrotic potential on rat glial cells [21]. To get further insights into *P. mosselii* virulence, we investigate in the present work the cytotoxicity and proinflammatory effects of two clinical strains of *P. mosselii* (ATCC BAA-99 and MFY161) on Caco2/TC7 cells, the transepithelial permeability of Caco2/TC7 monolayers and the actin network. The behavior of these bacteria was compared to that of the well-known opportunistic pathogen *P. aeruginosa* PAO1.

Results

Cytotoxicity assay

The cytotoxic effect of *P. mosselii* ATCC BAA-99 and MFY161 on Caco-2/TC7 cells was determined by quantification of lactate dehydrogenase (LDH) released in culture medium (Figure 1). The results show that *P. mosselii* ATCC BAA-99 was the less cytotoxic strain with only 30.1 +/-0.1% of cell lysis after 24 h of infection. *P. mosselii* MFY161 exhibited a cytotoxic activity reaching 64.5 +/-0.1% of lysis and the cytotoxic activity of *P. aeruginosa* PAO1 was higher with 85.6 +/-0.2% of lysis. Enumeration of *P. mosselii* ATCC BAA-99 (5×10^8 CFU.mL⁻¹), *P. mosselii* MFY161 (4.8×10^8 CFU.mL⁻¹) and *P. aeruginosa* PAO1 (4.9×10^8 CFU.mL⁻¹), at the end of the infection period showed that higher cytotoxicity was not due to bacterial overgrowth.

Bacterial invasion assay

The capacity of *P. mosselii* ATCC BAA-99 and MFY161 to enter Caco-2/TC7 cells has been investigated using the gentamicin exclusion test (Figure 2). The results show that the two *P. mosselii* strains studied can have an invasive behavior with $0.5 \pm 0.2 \times 10^5$ and $0.2 \pm 0.2 \times 10^5$ CFU.mL⁻¹ detected intracellularly for *P. mosselii* ATCC BAA-99 and MFY161, respectively. The invasive capacity of *P. aeruginosa* PAO1 was significantly higher with $1.4 \pm 0.1 \times 10^5$ CFU.mL⁻¹ that entered Caco-2/TC7 cells.

Quantification of IL-6, IL-8 and HBD-2 secretion

The bacterial proinflammatory effect of *P. mosselii* ATCC BAA-99, *P. mosselii* MFY161 and *P. aeruginosa* PAO1 was assessed by measuring IL-6 and IL-8 secretion in Caco-2/TC7 after 24 h of infection. The results show that the two strains of *P. mosselii* studied did not induce significant stimulation of IL-6 (Figure 3A) and IL-8 (Figure 3B) secretion in Caco-2/TC7 compared to uninfected cells. On the contrary, the infection of Caco-2/TC7 cells with *P. aeruginosa* PAO1 led to a major secretion of IL-8 with 92 ± 13 pg.mL⁻¹ (Figure 3B).

The human β -defensin-2 (HBD-2) was also quantified in Caco-2/TC7 cells supernatant. The results show that the two strains of *P. mosselii* were able to induce HBD-2 secretion by Caco-2/TC7 cells (Figure 3C). Infection with *P. mosselii* ATCC BAA-99 and MFY161 strains led to a major increase of HBD-2 production by Caco-2/TC7 with 125 ± 26 pg.mL⁻¹ and 136 ± 31 pg.mL⁻¹, respectively, compared to the 4 ± 2 pg.mL⁻¹ basal secretion of HBD-2 in uninfected cells. The induction of HBD-2 by the two *P. mosselii* strains was almost similar to that obtained with *P. aeruginosa* PAO1 (165 ± 14 pg.mL⁻¹).

Transepithelial electrical resistance measurements

The effect of the bacteria on epithelial permeability was evaluated by measuring the TER across differentiated

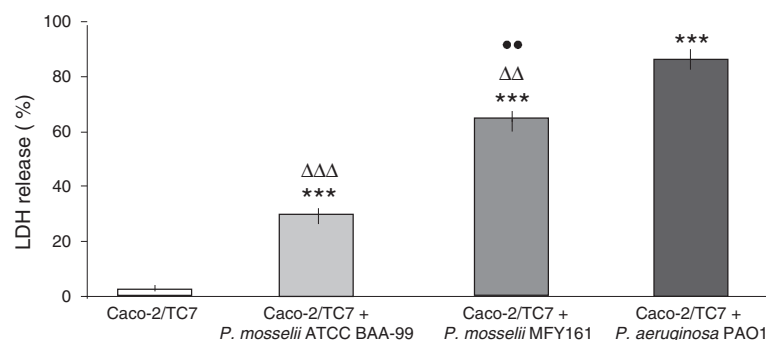


Figure 1 Cytotoxic effects of *P. mosselii* ATCC BAA-99, *P. mosselii* MFY161 and *P. aeruginosa* PAO1 on Caco-2/TC7 cells. Cytotoxicity was determined by LDH release assay after 24 h of infection. Results were calculated as the mean values (+/-SEM) of three independent experiments. *** P < 0.001 versus uninfected Caco-2/TC7 cells, ΔΔΔ P < 0.001 versus *P. aeruginosa* PAO1, ΔΔ P < 0.01 versus *P. aeruginosa* PAO1, ** P < 0.01 versus *P. mosselii* ATCC BAA-99.

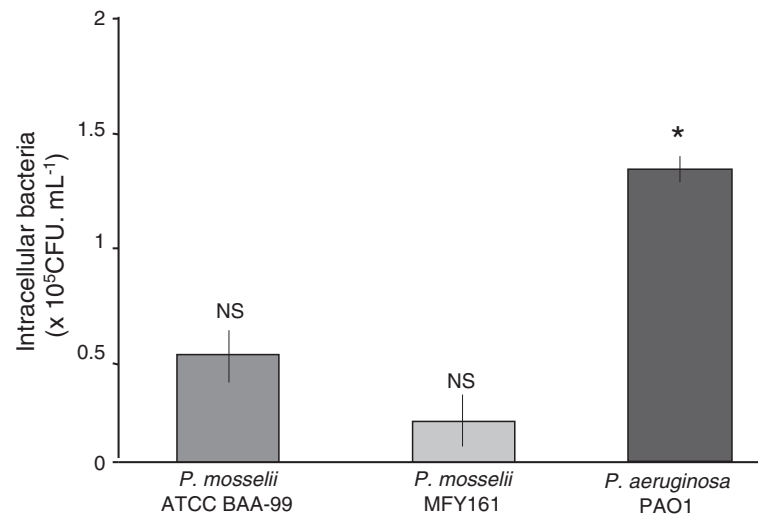


Figure 2 Invasive capacity of *P. mosselii* ATCC BAA-99, *P. mosselii* MFY161 and *P. aeruginosa* PAO1. 4 h after infection of Caco-2/TC7 cells with the bacteria, extracellular germs were killed by gentamicin. Cells were lysed and the intracellular bacteria were enumerated by plating onto nutrient agar medium. Results were calculated as the mean values (+/-SEM) of three independent experiments. * P < 0.05 versus *P. mosselii* ATCC BAA-99 and *P. mosselii* MFY161, NS not significant between *P. mosselii* ATCC BAA-99 and *P. mosselii* MFY161.

Caco-2/TC7 monolayers. TER values were measured at the onset of the experiment and at times 3, 6, 9 and 24 h. Up to 9 h after the beginning of the experiment, the TER values of the infected monolayers remained unchanged (data not shown). After 24 h of infection, the TER values of the monolayers exposed to the bacteria were significantly decreased (Figure 4). The decrease of TER induced by *P. mosselii* MFY161 was 20.8 +/-4.7% compared to uninfected control cells whereas *P. mosselii* ATCC BAA-99 led to a decrease of TER reaching 39 +/- 3.2% and *P. aeruginosa* PAO1 provoked a deeper decrease of the TER value (55.8 +/-5.3%). These falls in TER cannot be attributed to damages provoked by acidification of the medium since the pH of the medium remained constant over the studies.

Actin visualisation

The effect of *P. mosselii* ATCC BAA-99 and MFY161 on the organization of the sub-membrane F-actin microfilament network was studied and compared to that of *P. aeruginosa* PAO1. Whereas the staining pattern of untreated Caco-2/TC7 cells showed a continuous fine meshwork of microfilaments lining the cell border (Figure 5), the cells exposed for 24 h with *P. mosselii* ATCC BAA-99, *P. mosselii* MFY161 or *P. aeruginosa* PAO1 lost their normal organization. All these bacteria induced a dramatic disruption of F-actin.

Discussion

P. mosselii was formally described as a novel species in 2002 through a polyphasic taxonomic approach including 16SrDNA phylogeny, numerical analysis, DNA-DNA

hybridization, thermal stability of DNA-DNA hybrids and siderophore-typing methodology [19]. The several strains of *P. mosselii* described to date were isolated in hospital and some have been suggested as emerging human pathogens [19-21]. Our study aimed at investigating the virulence potential of two of these strains, namely ATCC BAA-99 and MFY161, belonging to the same cluster strongly related to the hospital-isolated *P. putida* on the basis of both *oprD* or *oprF*-linked phylogenies [22]. Although *P. putida* species is mostly known for its huge capacity in degradation of numerous carbon sources [23], some clinical strains have emerged, causing infections in immunosuppressed hosts and patients with invasive medical devices. More recently, *P. putida* has been involved in war wound infection, and should be considered as a potential human pathogen, for a review see Carpenter et al. [24].

In the present study, we further investigated the cytotoxicity of *P. mosselii* ATCC BAA-99 and MFY161 strains, and show that they provoked the lysis of the intestinal epithelial cells Caco-2/TC7, with a major damage obtained after infection with *P. mosselii* MFY161. The cytotoxic levels were lower compared to the well-known opportunistic pathogen *P. aeruginosa* PAO1 but almost similar to those observed for *P. mosselii* strains on rat glial cells [21], and for the clinical strain *P. fluorescens* MFN1032 on Caco-2/TC7 cells [17]. The gentamicin exclusion test showed that *P. mosselii* ATCC BAA-99 and MFY161 can enter Caco-2/TC7 cells. The invasion capacity of the two *P. mosselii* strains studied was similar and lower than that of the pathogen *P. aeruginosa* PAO1.

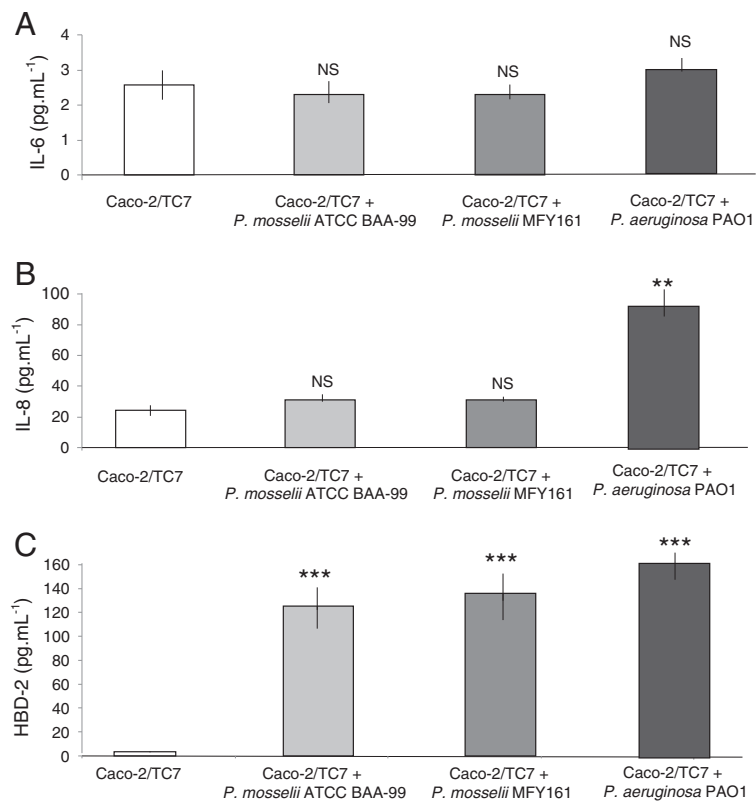


Figure 3 Proinflammatory effects of *P. mosselii* ATCC BAA-99, *P. mosselii* MFY161 and *P. aeruginosa* PAO1 on Caco-2/TC7 cells. IL-6 and IL-8 cytokines, and HBD-2 were measured in Caco-2/TC7 cells supernatant after 24 h of infection. Results were calculated as the mean values (+/-SEM) of three independent experiments. *** P < 0.001 versus uninfected Caco-2/TC7 cells, ** P < 0.01 versus uninfected Caco-2/TC7 cells, NS not significant.

The bacterial proinflammatory effect of *P. mosselii* ATCC BAA-99 and MFY161 was then assessed by measuring the secretion of IL-6 and IL-8 cytokines in Caco-2/TC7 after 24 h of infection. The results showed that the two strains did not induce the production of these proinflammatory cytokines. We hypothesize that this

may serve as a strategy for *P. mosselii* to escape the immune system. However, *P. mosselii* ATCC BAA-99 and MFY161 were found to strongly increase the secretion of HBD-2. Human beta-defensins are known to play a key role in host defense. In fact, in addition to their potent antimicrobial properties against commensal and

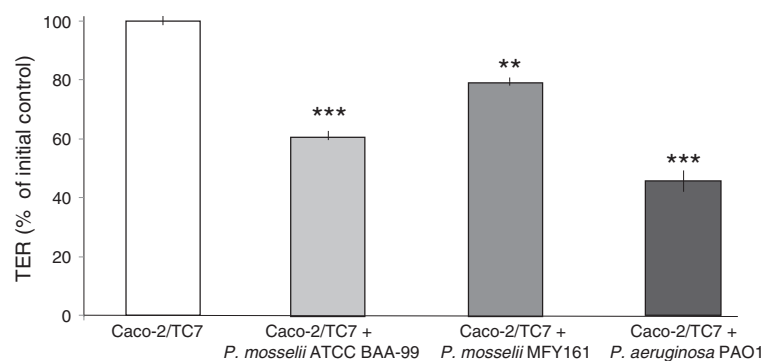


Figure 4 Effects of *P. mosselii* ATCC BAA-99, *P. mosselii* MFY161 and *P. aeruginosa* PAO1 on the transepithelial electrical resistance of Caco-2/TC7 cells. Differentiated Caco-2/TC7 cells were infected for 24 h. The TER was expressed as percentages of the initial control TER measured across each individual cell monolayer at the onset of the experiment. Results are the mean values (+/-SEM) of three independent experiments. *** P < 0.001 versus uninfected Caco-2/TC7 cells, ** P < 0.01 versus uninfected Caco-2/TC7 cells.

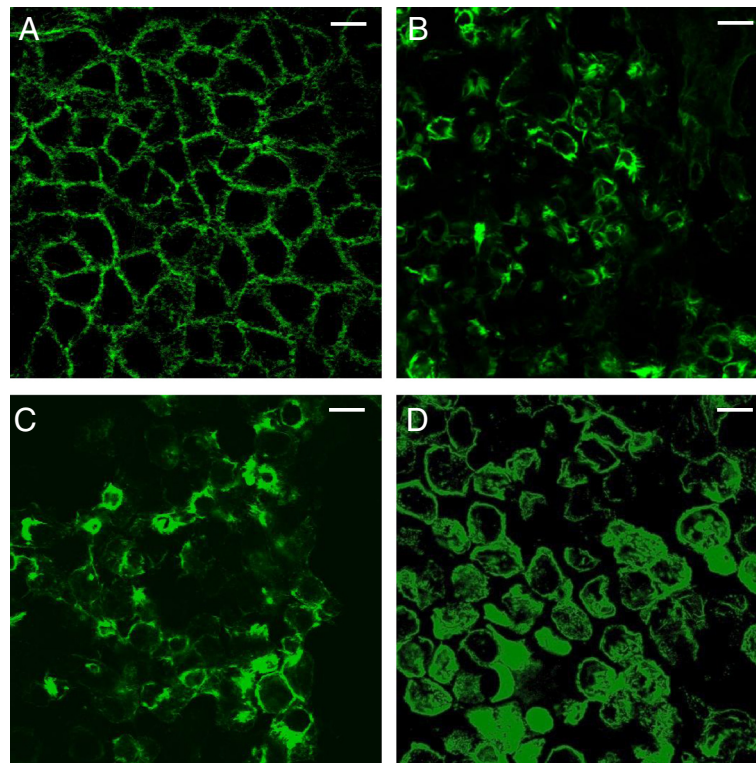


Figure 5 Effects of *P. mosselii* ATCC BAA-99, *P. mosselii* MFY161 and *P. aeruginosa* PAO1 on the F-actin cytoskeleton of Caco-2/TC7 cells. Differentiated Caco-2/TC7 cells were infected for 24 h. F-actin was stained and examined using a confocal laser scanning microscope. (A) Uninfected cells, (B) infection by *P. mosselii* ATCC BAA-99, (C) infection by *P. mosselii* MFY161, (D) infection by *P. aeruginosa* PAO1. Scale bar 100 μm .

pathogenic bacteria [25], beta-defensins were demonstrated to function as multieffector molecules capable of enhancing host defense by recruiting various innate as well as adaptive immune cells to the site of infection. Nevertheless, some pathogens can be resistant to HBD-2 [26] and surprisingly can induce and divert HBD-2 secretion in intestinal epithelial cells to enhance its capacity of virulence [27].

The effect of *P. mosselii* ATCC BAA-99 and MFY161 on epithelial permeability was then evaluated by measuring the TER across differentiated Caco-2/TC7 monolayers. The F-actin cytoskeleton was stained with Alexa-488 phalloidin and examined using a confocal laser scanning microscope. We observed that the TER of the monolayers exposed to the bacteria was significantly decreased and that the F-actin cytoskeleton was completely broken. Similar results of TER decrease and F-actin disruption were previously observed with many pathogens including *Salmonella typhimurium*, *P. aeruginosa* and *Escherichia coli* [28-30].

Infections caused by multidrug-resistant (MDR) Gram-negative bacilli have become a growing challenge in hospital [31]. In a recent study, Giani et al. [32] suggested that unusual human opportunistic pathogen like *P. mosselii* may probably play a role as shuttles for acquired

metallo- β -lactamases resistance thus an antibiogram was made for *P. mosselii* ATCC BAA-99 and MFY161 (see Additional file 1: Table S1). We found that the two strains were resistant towards 6 of the 16 antibiotics tested including the ticarcillin beta-lactam, which could support the above hypothesis.

Conclusion

In conclusion, our study demonstrates that *P. mosselii* ATCC BAA-99 and MFY161 are cytotoxic towards Caco-2/TC7 cells, have low invasive capacity, induce secretion of human β -defensin 2 (HBD-2), alter the epithelial permeability of differentiated cells and damage the F-actin cytoskeleton. These strains are less virulent than *P. aeruginosa* PAO1, but their behavior resembles that of cytotoxic strains of *P. fluorescens* [17,18] and by thus may be considered as potential emerging human pathogen.

Methods

Bacterial strains

P. mosselii ATCC BAA-99 is a clinical strain isolated from tracheal aspirate of a patient suffering from pulmonary infections [19]. *P. mosselii* MFY161 was collected from urine of a patient suffering from alcoholic hepatitis in Charles Nicolle hospital (Rouen, France), and characterized

by 16SrDNA, *oprF* and *oprD* sequencing [7,8], and siderotyping [22]. *P. aeruginosa* PAO1 was obtained from an international collection. All the strains were routinely cultivated under vigorous shaking, in ordinary nutrient broth (Merk, Darmstadt, Germany), at their optimal growth temperature, 30°C for *P. mosselii* ATCC BAA-99 and MFY161, 37°C for *P. aeruginosa* PAO1.

Cell line and culture

Caco-2/TC7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 15% of heat-inactivated fetal calf serum, 2 mM of L-glutamine, 100 U.mL⁻¹ each of penicillin and streptomycin and 1% of non-essential amino acids. For the experimental assays, the cells were seeded at a density of 10⁵ cells.cm⁻² in 24-wells tissue culture plates, or on inserts (6.4 mm diameter, 3 µm pore size, Falcon) to obtain fully differentiated cells. The cells were cultured at 37°C in 5% CO₂-95% air atmosphere and the medium was changed daily.

Cell infection

Bacteria of overnight culture were harvested by centrifugation (5000 g, 5 min, 20°C) and resuspended at a density of 10⁸ colony forming unit (CFU).mL⁻¹ in cell culture medium without serum and antibiotics. Caco-2/TC7 cells grown on 24-wells culture plates or inserts were washed twice with fresh culture medium and the bacterial suspensions were applied to the cell surface at a concentration of 10⁸ CFU.cm⁻², resulting to a multiplicity of infection (MOI) of 100. Infected cells were then incubated at 37°C in 5% CO₂-95% air during 24 h for all experiments, excepted 4 h of infection for the invasion test. Each assay was conducted in triplicate in independent experiments (successive passages of Caco-2/TC7 cells).

Cytotoxicity assay

Cytotoxicity assay was performed on confluent Caco-2/TC7 grown in 24-wells culture plates. After 24 h of infection, the supernatants from Caco-2/TC7 monolayers were collected and the concentration of lactate dehydrogenase (LDH), a cytoplasmic enzyme released upon cell death, was determined using an enzymatic assay (Cytotox 96 Promega, Charbonnières, France) as previously described [17]. Caco-2/TC7 cells exposed to Triton ×100 (0.9%) were used as a control of total LDH release (100% dead cells).

Bacterial invasion assay

After 4 h of infection, Caco-2/TC7 monolayers were washed with phosphate-buffered saline (PBS). Adherent bacteria were killed by incubation for 1 h with 300 µg.mL⁻¹ gentamycin, an antibiotic that does not cross the cytoplasmic membrane of eukaryotic cells and then only kills bacteria not internalized in cells. Caco-2/TC7

monolayers were washed 3 times with PBS to remove the antibiotic and dead bacteria. The cells were then lysed by incubation for 15 min with 0.5% Triton ×100 to release the intracellular bacteria and the lysates were plated onto nutrient agar to determine the number of internalized bacteria.

Quantification of IL-6, IL-8 and HBD-2

After 24 h of infection with the bacterial suspensions, the levels of IL-6 and IL-8 cytokines were measured in Caco-2/TC7 cells supernatant using ELISA Quantikine kits (R&D systems). The human β-defensin-2 (HBD-2) was quantified using the Defensin 2, beta (Human) - ELISA Kit (Phoenix Pharmaceuticals inc). These assays were conducted according to the manufacturer's protocols.

Transepithelial electrical resistance measurements

Caco-2/TC7 cells grown on inserts were used at 21 days post-confluence (fully differentiated cells) and the transepithelial electrical resistance (TER) of the monolayers infected or not with the bacterial strains was measured during 24 h using the Millicell Electrical Resistance System (Millipore Corp, Bedford, MA). TER values are expressed as percentages of the pre-infection level of the TER (baseline) measured for each individual cell monolayer in the inserts.

Actin visualisation

Fully differentiated Caco-2/TC7 monolayers were exposed to the bacterial strains for 24 h. At the end of the experiment, the cells were washed with PBS, fixed for 10 min in 3.7% paraformaldehyde and permeabilized for 5 min with 0.1% Triton ×100 at room temperature. The cells were then incubated with 1% bovine serum albumin in PBS for 10 min and the apical F-actin cytoskeleton was stained with Alexa-488 phalloidin (1U/insert) for 45 min at room temperature. Following three washes in PBS, cell monolayers were examined using a confocal laser scanning microscope (Zeiss, LSM710).

Statistical analysis

All experiments were conducted independently at least three times. The results are expressed as means +/- SEM and statistical significance were performed by Student's *t*-test.

Additional file

Additional file 1: Table S1. Antibiotic susceptibility pattern of *P. mosselii* ATCC BAA-99 and *P. mosselii* MFY161. The antibiotics tested were ticarcillin (TIC), piperacillin (PRL), colistin (CT), imipenem (IPM), aztreonam (ATM), tobramycin (TOB), gentamycin (GN), amikacin (AK), ticarcillin + clavulanic acid (TIM), ceftazidime (CAZ), ciprofloxacin (CIP), cefsulodin (CFS), levofloxacin (LEV), trimethoprim-sulphamethoxazole (SXT), fosfomycin (FF) and netilmicine (NET). R, resistant; I, intermediate; S, susceptible.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EB, MF, SC and NC designed the experiments, supervised the research and wrote the paper. CLJ, AM, KB and NC did the experiments and/or data analysis. All authors read and approved the final manuscript.

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-Article 5-

ORIGINAL ARTICLE

Pseudomonas fluorescens Alters the Intestinal Barrier Function by Modulating IL-1 β Expression Through Hematopoietic NOD2 Signaling

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Pseudomonas fluorescens Alters the Intestinal Barrier Function by Modulating IL-1 β Expression Through Hematopoietic NOD2 Signaling

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Background: Ileal Crohn's disease is related to *NOD2* mutations and to a gut barrier dysfunction. *Pseudomonas fluorescens* has also been associated with ileal Crohn's disease. The aim of this study was to determine the impact of *P. fluorescens* on the paracellular permeability in ileum and Peyer's patches.

Methods: To explore this question, in vivo and ex vivo experiments were performed in wild-type, *Nod2*^{-/-}, *Nod2*^{2939iC}, and *IL-1R*^{-/-} mice together with in vitro analyses using the Caco-2 (epithelial) and the THP-1 (monocyte) human cell lines.

Results: *Pseudomonas fluorescens* increased the paracellular permeability of the intestinal mucosa through the secretion of IL-1 β by the immune cell populations and the activation of myosin light chain kinase in the epithelial cells. Induction of the IL-1 β pathway required the expression of *Nod2* in the hematopoietic compartment, and muramyl dipeptide (a *Nod2* ligand) had an inhibitory effect.

Conclusions: *Pseudomonas fluorescens* thus alters the homeostasis of the epithelial barrier function by a mechanism similar to that previously observed for *Yersinia pseudotuberculosis*. This work further documents a putative role of psychrotrophic bacteria in Crohn's disease.

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Key Words: paracellular permeability, *Pseudomonas fluorescens*, *Nod2*, IL-1 β , Myosin light chain kinase

The intestinal mucosa harbors the highest number and diversity of permanently associated microorganisms.¹ Interactions between the mucosal immune system, the epithelial monolayer, and commensal bacteria elicit basal level immune responses in normal individuals while a defect of the gut barrier is associated with gut inflammation as in the case of Crohn's disease (CD).²

CD is a complex genetic disorder that results in an inappropriate gut immune response, an impaired epithelial barrier

function, and a dysbiosis.^{1,3,4} CD lesions are related to gut-associated lymphoid tissue.^{5,6} Lymphoid follicles are mainly encountered in the colon where they are isolated and in small bowel where they are grouped forming Peyer's patches (PP). Lymphoid follicles are known to be pivotal sites for the host immune response and for the entry of enteropathogenic bacteria.⁷

Barrier defects are reflected by increased paracellular permeability (ParaP) in patients with CD and their healthy asymptomatic first-degree relatives.⁸ The increased ParaP may precede clinical evidence of disease and can serve as a marker of impending disease reactivation. Finally, it correlates with systemic immune activation.^{9,10}

Among the more than 100 known CD-associated polymorphisms, *NOD2* mutations exhibit the highest risks and the highest specificities.^{11,12} They are more frequently found in ileal CD.¹² *NOD2* belongs to the nucleotide-binding domain and leucine-rich repeat family of cytoplasmic proteins.¹³ When stimulated by muramyl dipeptide (MDP), a moiety of peptidoglycan, *NOD2* activates several proinflammatory pathways including NF- κ B, MAP kinases, and caspase 1.^{14,15} Because MDP is conserved in both gram-positive and gram-negative bacteria, *NOD2* is supposed to detect a wide variety of microorganisms.¹⁶

Nod2 is involved in the regulation of microbiota and the gut homeostasis.^{17–20} Under basal conditions, *Nod2* knockout (*Nod2*^{-/-}) mice are characterized by a hypertrophy of the

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gut-associated lymphoid tissue in presence of the gut microbiota.¹⁹ In more detail, *Nod2* modulates the cross talk between T CD4⁺ cells and the epithelial cells of the follicle-associated epithelium covering PP and thus maintains the homeostasis of the intestinal barrier function.¹⁹ In human, CD patients with *NOD2* mutations have altered epithelial cell–cell contacts evidenced by an increase of intestinal ParaP.^{21,22} Despite a higher basal ParaP, when infected with *Yersinia pseudotuberculosis*, *Nod2*^{-/-} mice limit the early phases of bacterial colonization of the gut mucosa.²³

Nod2^{-/-} mice do not develop spontaneous ileocolitis, suggesting that *Nod2* dysregulation alone is not sufficient to induce CD.¹⁸ Thus, environmental risk factors linked with the modern western way of life are supposed to be required for the development of CD.^{1,3,24} Among them, a relation between exposure to psychrotrophic bacteria and CD is suggested by the parallel emergence of the disease in western countries and the development of refrigeration.^{25,26} Thus, *Yersinia*, *Listeria*, or *Pseudomonas* has been suspected to play a role in CD.^{27–29} It is to note that *Yersinia* and *Listeria* species exhibit a tropism for PP by invading the M cells present in the follicle-associated epithelium of the PP, and they are able to alter the ParaP of the ileal mucosa.^{23,30–33}

Pseudomonas fluorescens is present at low numbers in the intestinal lumen and in many ecological niches including soil and water but also in refrigerated food products where it can grow.³⁴ Although, *P. fluorescens* has long been considered as a psychrotrophic microorganism, some clinical strains have been able to adapt at a growth temperature of 37°C.³⁵ Recently, clinical strains of *P. fluorescens* were shown to increase the ParaP and induce cytotoxic and cytokine responses in Caco-2/TC7 cells.^{36–38} This event confers an increase of virulence, in the range of the well-known opportunistic pathogen *P. aeruginosa*. Furthermore, a highly specific antigen of *P. fluorescens*, designated as I2, was detected in the serum of 54% of patients with CD, and a correlation between the severity of the disease and the level of the circulating I2 antigen has been evidenced.^{29,39} Moreover, the increase of I2 levels in serum is linked with the presence of CD lesions in the ileum.²⁹ Nevertheless, the effect of *P. fluorescens* on the destabilization of the gut barrier function has not been studied in vivo. Here, we have investigated the molecular mechanisms by which *P. fluorescens* alters the intestinal epithelial barrier with specific reference to *Nod2* function.

MATERIALS AND METHODS

Animals

All the animals were on the C57BL/6 background. Wild-type (WT), *Nod2*-deficient (*Nod2*^{-/-}), *Nod2*^{2939iC} mice (carrying the mutation homologous to the 3020insC human mutation), and interleukin (IL)-1 receptor deficient (*IL-1R*^{-/-}) mice were generated in the animal facility at Robert Debré Hospital.^{18,19,40} All mice were housed in pathogen-free conditions with free access to food and water. Pathogen-free conditions were monitored every 6 months in accordance with the full set of Federation of Laboratory Animal

Science Associations high standards recommendations. Housing and experiments adhered to institutional animal health care guidelines, and experiments were approved by the institutional committee for animal use.

Bacterial Strains and Growth Condition

P. fluorescens MFN1032 (biovar I) was collected in a hospital of Haute-Normandie (France). It was characterized by polyphasic identification, 16S RNA sequencing, and siderotyping.³⁵ *P. fluorescens* MF37 is a spontaneous rifampicin-resistant mutant of the strain MF0 (biovar V), originally identified in crude milk.⁴¹ *P. aeruginosa* PAO1 was obtained from an international collection. All the strains were cultivated in an ordinary nutrient broth, at 28°C for the 2 strains of *P. fluorescens* or 37°C for *P. aeruginosa* PAO1.

Cell Lines

Caco-2 cells were purchased from the DSMZ collection (Braunschweig, Germany). Caco-2 cells were grown at 37°C in a 5% CO₂ water-saturated atmosphere in GlutaMAX DMEM (Gibco, Saint Aubin, France) supplemented with 10% of heat-inactivated fetal bovine serum (Biowest, Nuaillé, France), 1% nonessential amino acids, and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin; Gibco). Cells were seeded in Transwell inserts (CoStar, Paris, France) and grown for 14 days. THP-1 cells were obtained from the ATCC collection (Molsheim, France). They were grown at 37°C in a 5% CO₂ water-saturated atmosphere in RPMI-1640 (Gibco) containing 10% heat-inactivated fetal bovine serum, 1% L-glutamine (Gibco) supplemented with 1% antibiotics, and 0.01% of β-mercaptoethanol (Sigma, St. Quentin Fallavier, France).

Bone Marrow-derived Macrophagic Cells

Macrophagic cells (MΦ) and bone marrow stem cells (BMSC) were cultured for 6 days in a medium supplemented with macrophage colony-stimulating factor containing supernatants from the L929 cell line.⁴²

Animal Infections

Mice were intragastrically (ig) inoculated with a single dose of *P. fluorescens* MFN1032 or *P. aeruginosa* PAO1 (10⁸ colony-forming unit [CFU]/100 μL of sterile water). At day 7, gut permeability and cytokine concentrations were assessed in the ileal mucosa and in PP. To test the effect of IL-1β and myosin light chain kinase (MLCK), mice were intraperitoneally pretreated with Anakinra (300 μg per mice per d), Z-YVAD-FMK (100 μg per mice per d; Kamiya Biomedical, Seattle, WA), MDP (100 μg per mice per d; Sigma), and ML-7 (2 mg/kg; Sigma), respectively, for 2 consecutive days before experimentation.

Ex vivo Infection

After mouse was killed, PP were mounted in Ussing chamber (UC). 10⁸ CFU of *P. fluorescens* MFN1032 or MF37 strains or *P. aeruginosa* PAO1 were added into the mucosal compartment. When indicated, Anakinra (50 μg/mL), Z-YVAD-FMK (20 μg/mL), MDP (10 μg/mL), and ML-7 (20 μg/mL), or alternatively

the same amount of vehicles, were used in the mucosal compartment of UC.

Cell Infections

MΦ or THP-1 cells were infected by adding *P. fluorescens* MFN1032 or *P. aeruginosa* PAO1 strains (multiplicity of infection [MOI], 10). After 2, 6, or 24 hours, extracellular bacteria were killed by gentamycin (100 μg/mL; Sigma), and cytokine (IL-1β, IL-8, IL-12, tumor necrosis factor [TNF]-α, or interferon γ) levels were measured by enzyme-linked immunosorbent assay in the supernatants.

Measures of Permeability

In Vivo Experiments

Mice were gavaged with fluorescein isothiocyanate-labeled dextran 4 kDa (FD4; 15 mg/100 μL per mice; Sigma) 6 hours before the sacrifice. Whole serum FD4 levels were determined with a fluorometer (PerkinElmer, Courtaboeuf, France).

Ex Vivo Experiments

After mouse was killed, biopsies from ileal mucosa or PP were mounted in UC and maintained in circulating oxygenated Ringer's solution at 37°C throughout the experiment. ParaP was assessed 2 hours after infection by measuring the mucosal-to-serosal flux of FD4.

Coculture Experiments

FD4 (10^{-5} M) were added into the apical compartment of the transwell system (TS). The permeability was monitored by sampling 400 μL from the basolateral compartment 24 hours after addition of 10^6 THP-1 cells, infected or not by *Pseudomonas*, in the basolateral chamber. When indicated, Caco-2 cells were preincubated 24 hours with Anakinra (50 μg/mL), MDP (10 μg/mL), or ML-7 (20 μg/mL).

Chimeric Mice

BMSC were isolated from WT Ly5.1 or *Nod2*^{-/-} Ly5.2 mice. Five million cells were injected intravenously either into *Nod2*^{+/+}Ly5.1 or *Nod2*^{-/-}Ly5.2 lethally irradiated recipients. Chimerism was assessed at week 12 by flow cytometry using the Ly5.1 and Ly5.2 markers.

Cytokines

In vivo, 7 days after infection, PP and ileal mucosa from WT, *NOD2*^{-/-}, and *IL-1R*^{-/-} mice were removed, washed, and homogenized in phosphate-buffered saline containing protease inhibitor (Roche, Boulogne-Billancourt, France), and IL-1β levels were measured by enzyme-linked immunosorbent assay (BD Biosciences, Heidelberg, Germany). In vitro, cytokine concentrations were determined in the supernatant 2, 6, and 24 hours after infection of macrophages (MOI, 10) and THP-1 cells (MOI, 10).

Real-Time PCR

After extraction by the NucleoSpin RNA II Kit (Macherey-Nagel, Hoerdt, France), total RNA was converted to complementary

DNA using random hexonucleotides. Polymerase chain reaction was performed using QuantiTect SYBR Green PCR Kit (Applied Biosystems, Saint Aubin, France), sense and antisense primers specific for Claudin-2, Claudin-5, Claudin-8, Occludin, and G3PDH and for the long isoform of MLCK. Sequences are shown in Table, Supplemental Digital Content 1, <http://links.lww.com/IBD/A689>. After amplification, we determined the cycle threshold (Ct) to obtain expression values of $2^{-\Delta\Delta Ct}$.

Small Interfering RNAs

The ON-TARGET plus SMARTpool small interfering RNAs targeting NOD2, Rick, TLR-2 (L-005120-01-0005), and TLR-4 (L-008088-01) and nontargeted control small interfering RNA were purchased from Dharmacon (Lafayette, CO). THP-1 cells were transfected according to manufacturer's instructions (Invitrogen, Cergy Pontoise, France). Transfections were done 48 hours before experiments as it has been previously shown in Refs. 23 and 30.

MLCK Protein

Caco-2 cells were washed with phosphate-buffered saline and lysed. MLCK concentrations were determined by enzyme-linked immunosorbent assay (Cusabio, Paris, France) according to the manufacturer's instructions. For more details, see article in Ref. 30.

Statistical Analysis

Multigroup comparisons were performed using a 1-way analysis of variance followed by a Bonferroni correction for multiple tests. Two-group comparisons were performed using an unpaired *t* test assuming the Gaussian distribution. The Gaussian distribution was tested by Kolmogorov-Smirnov test. Statistical analyses were performed using GraphPad Prism 4.00 (GraphPad software, San Diego, CA). A value of $P < 0.05$ was considered statistically significant. All *P* values were 2-sided.

Study Approval

Housing and experiments were led following institutional animal healthcare guidelines and were approved by the local ethical committee for animal experimentation (Comité Régional d'Éthique en matière d'Expérimentation Animale n°4).

RESULTS

Pseudomonas Species Alter the Gut ParaP

P. fluorescens MFN1032 (a clinical strain), *P. fluorescens* MF37 (originally identified in crude milk), and *P. aeruginosa* PAO1 have previously been reported to increase the ParaP of polarized Caco-2/TC7 cells.³⁸ To determine whether *Pseudomonas* species also alters the intestinal barrier in vivo, C57BL/6j mice were ig inoculated with 10^8 CFU of *P. fluorescens* MFN1032, *P. fluorescens* MF37, or *P. aeruginosa* PAO1. Seven days after infection, 4 kDa of FITC-dextran (FD4) was ig inoculated and the marker concentration was measured in the serum. The levels of FD4 were higher after *Pseudomonas* infection than in uninfected mice, demonstrating an increase of ParaP (Fig. 1A). To investigate the

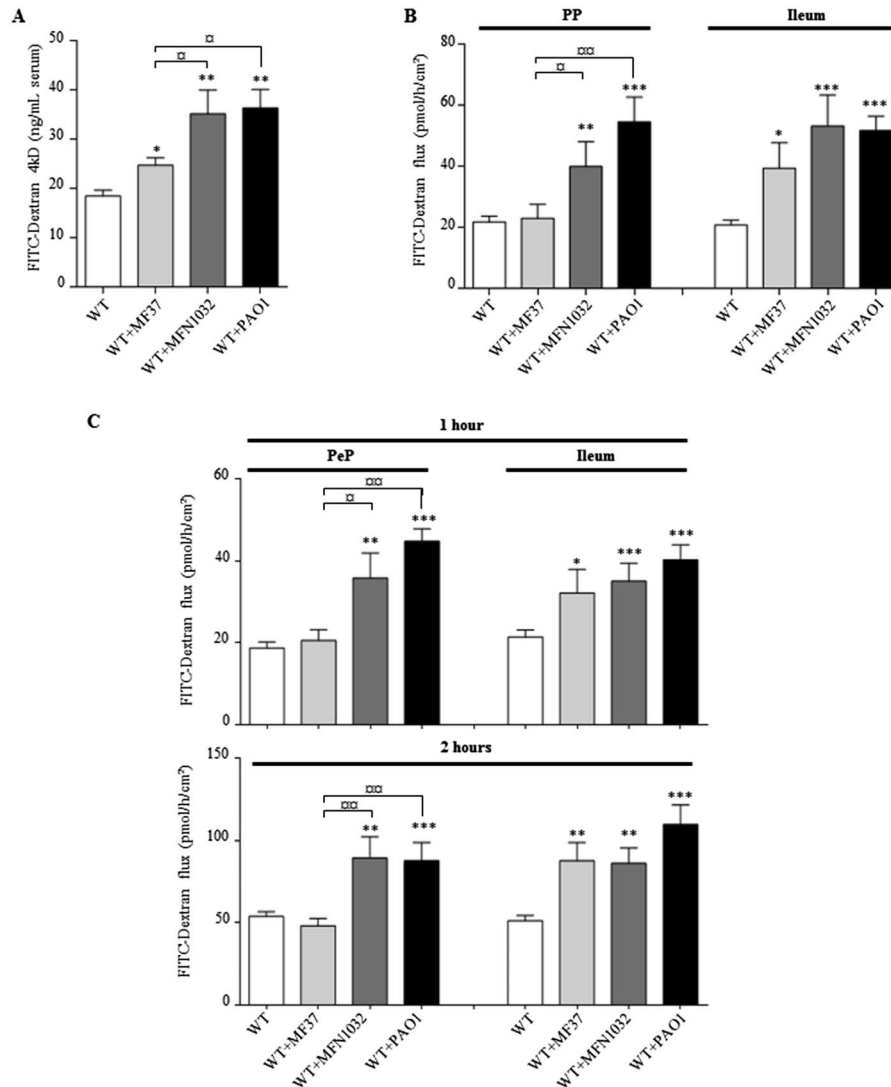


FIGURE 1. *Pseudomonas* alters the ParaP of the intestinal mucosa. A and B, WT mice were ig inoculated with 10^8 CFU of *P. fluorescens* MF37 or MFN1032 strains or *P. aeruginosa* PAO1 during 7 days. A, ParaP was investigated by measuring the accumulation of dextran-FITC 4 kDa (FD4) in the serum 6 hours after intragastrical exposure. B, Biopsies from PP or ileal mucosa were mounted in UC, and the ParaP was measured for 1 hour. C, Biopsies of PP or ileal mucosa from WT uninfected mice were mounted in UC and exposed to 10^8 CFU of different strains of *P. fluorescens* or *P. aeruginosa*. Then, ParaP was measured by monitoring the flux of FD4 for 1 or 2 hours. At least $n = 10$ per group; 3 independent experiments; mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT uninfected mice; $\square P < 0.05$ and $\square\square P < 0.01$ versus MF37 group.

segment of the intestine mostly affected by *Pseudomonas* infection, UC experiments were performed on ileal biopsies centered or not by PP formations. Biopsies were collected 7 days after intragastrical inoculation. The ParaP of both PP and ileal mucosa was higher in WT mice infected with *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 compared with controls (Fig. 1B). In contrast, *P. fluorescens* MF37 altered only the ParaP outside of PP formations (Fig. 1B). Finally, similar conclusions were drawn when biopsies obtained from uninfected mice were exposed to *Pseudomonas* species ex vivo and tested in UC (Fig. 1C). Altogether, these data evidenced that *Pseudomonas* species alter the gut barrier of the small intestine.

Pseudomonas Species Trigger IL-1 β and TNF- α Synthesis

Cytokine production may affect the function of the epithelial barrier.^{43,44} Thus, the levels of IL-1 β and TNF- α in PP and ileal biopsies were determined 7 days after infection. IL-1 β and TNF- α levels were increased after infection by *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 (Fig. 2A). *P. aeruginosa* PAO1 also increased the levels of interferon γ and IL-12 (see Fig. A and B, Supplemental Digital Content 2, <http://links.lww.com/IBD/A690>).

To determine the cytokine production in response to *Pseudomonas* in vitro, BMSC from WT mice were

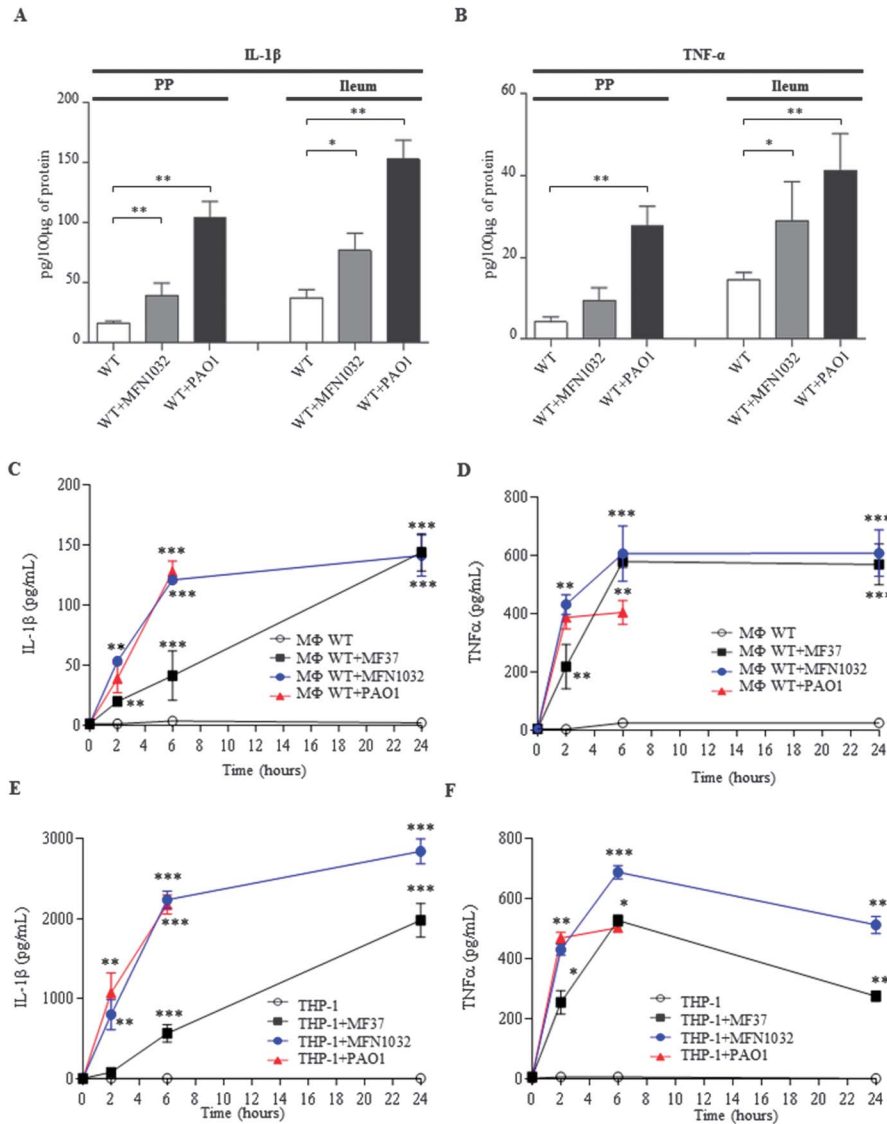


FIGURE 2. *Pseudomonas* triggers IL-1 β and TNF- α synthesis. A and B, WT mice were ig inoculated with 10⁸ CFU of *P. fluorescens* MFN1032 or *P. aeruginosa* PAO1 and killed 7 days after infection. A and B, Biopsies of PP or ileal mucosa were removed and (A) IL-1 β and (B) TNF- α levels were measured by enzyme-linked immunosorbent assay. C and D, Bone marrow stem cells from WT mice were differentiated into M Φ cells and infected (MOI = 10) with *P. fluorescens* MFN1032, *P. fluorescens* MF37, or *P. aeruginosa* PAO1 for 2, 6, or 24 hours. Then (C) IL-1 β or (D) TNF- α levels into the supernatant were assessed by enzyme-linked immunosorbent assay. E and F, THP-1 cells were exposed (MOI = 10) to *P. fluorescens* MFN1032, *P. fluorescens* MF37, or *P. aeruginosa* for 2, 6, or 24 hours. After centrifugation, the levels of (E) IL-1 β or (F) TNF- α were measured in the supernatants by enzyme-linked immunosorbent assay. At least n = 10 per group; 3 independent experiments; mean \pm SEM; *P < 0.05, **P < 0.01, and ***P < 0.001 versus control.

differentiated into macrophagic cells (M Φ). Then M Φ were infected (MOI = 10) with *Pseudomonas* species for 2, 6, and 24 hours. Since 2 hours after infection by *P. fluorescens* MFN1032 or *P. aeruginosa* PAO1, IL-1 β and TNF- α levels increased (Fig. 2C, D). Similarly, 2 hours after infection (MOI = 10) of the human monocyte-derived THP-1 cell line, *P. fluorescens* MFN1032 and *P. aeruginosa* PAO1 induced the secretion of IL-1 β and TNF- α (Fig. 2E, F). Most often, the effect of *P. fluorescens* MF37 was less marked (Fig. 2C, D). Six hours after infection, *P. aeruginosa* PAO1 killed more

efficiently M Φ and THP-1 cells than *P. fluorescens* MFN1032 (data not shown).

IL-1 β Expression Induced by *P. fluorescens* MFN1032 Disrupts Intestinal Barrier Function

Because IL-1 β is known to increase the gut ParaP,⁴³ to document its role in the case of *Pseudomonas* infection, WT or IL-1 receptor knockout (*IL-1R*^{-/-}) mice were ig inoculated with *P. fluorescens* MFN1032 during 7 days. In vivo, *P. fluorescens* MFN1032 did not alter the ParaP in *IL-1R*^{-/-} mice (Fig. 3A).

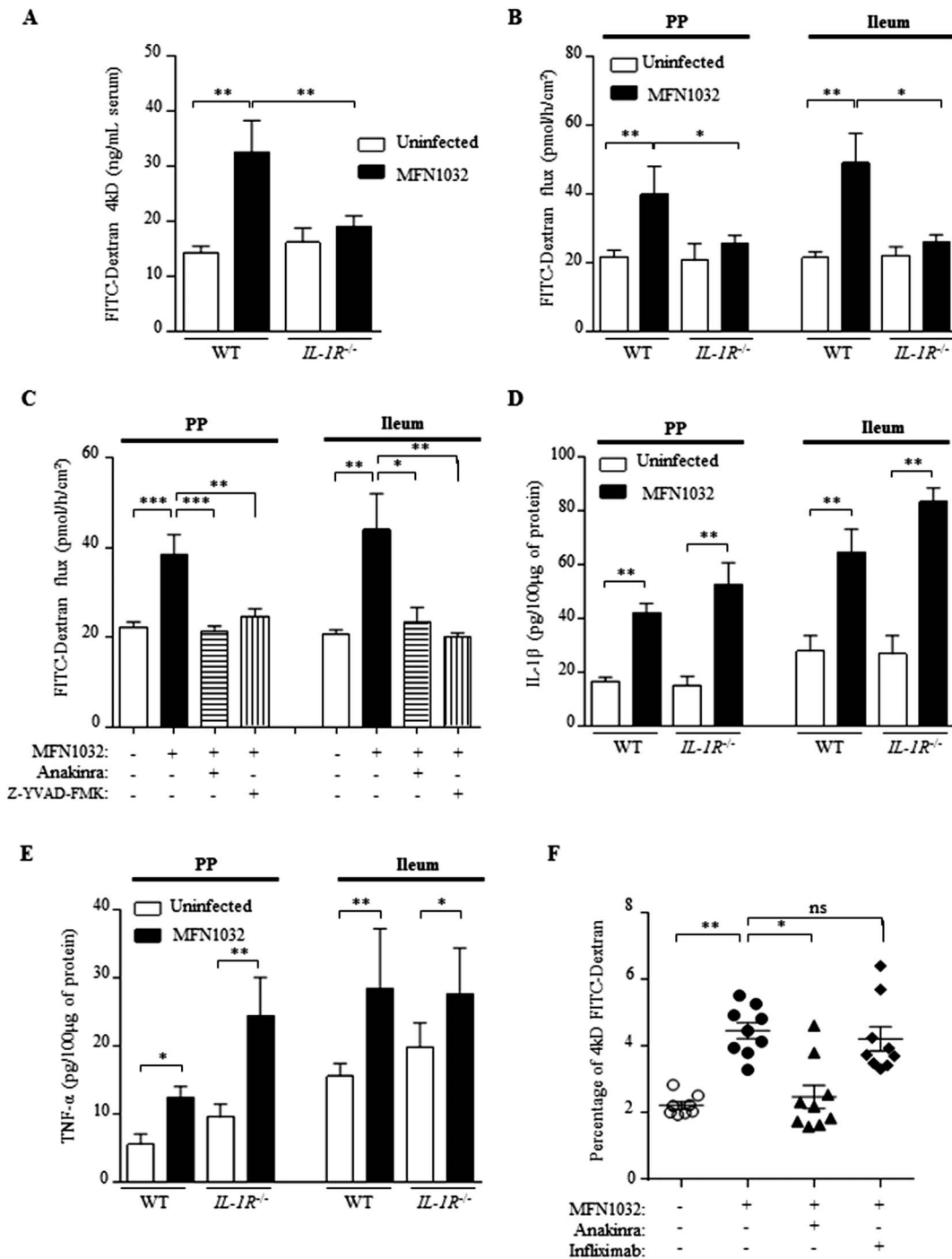


FIGURE 3. IL-1 β expression induced by *P. fluorescens* MFN1032 disrupts intestinal barrier function. A–D, WT or *IL-1R^{-/-}* mice were ig inoculated with 10⁸ CFU of *P. fluorescens* MFN1032 for 7 days. A, ParaP was investigated by measuring the quantity of FD4 in the serum. B, After mice were killed, biopsies of PP or ileal mucosa were mounted in UC, and the ParaP was measured for 1 hour. C, Biopsies of PP or ileum from uninfected WT mice were mounted in UC and incubated with *P. fluorescens* MFN1032. Where indicated, WT mice were intraperitoneally treated with Anakinra (an IL-1 receptor antagonist; 300 μ g/mice) or with Z-YVAD-FMK (a caspase-1 inhibitor, 100 μ g/mice) 2 days before experimentation. During experimentation, Anakinra (50 μ g/mL) or Z-YVAD-FMK (20 μ g/mL) was added into UC. Then, ParaP was measured for 1 hour. D, IL-1 β and (E) TNF- α levels were measured by enzyme-linked immunosorbent assay in PP or in ileal mucosa. F, Caco-2 cells were cultivated on TSs. THP-1 cells infected by *P. fluorescens* MFN1032 for 6 hours were then added into the basolateral compartment, and ParaP was monitored. To investigate the involvement of IL-1 β receptor or TNF- α in the increase of ParaP, Caco-2 cells were treated for 24 hours with Anakinra (50 μ g/mL) or infliximab (100 μ g/mL) (data are expressed as mean \pm SEM; * P < 0.05, ** P < 0.01, and *** P < 0.001; n = 8–10 per group; 3 separate experiments).

Measurements of the FD4 fluxes in UC experiments after in vivo or ex vivo exposures confirmed that IL-1R is required for the effect of *P. fluorescens* MFN1032 (Fig. 3B and see Fig. A, Supplemental Digital Content 3, <http://links.lww.com/IBD/A691>). WT mice infected with *P. fluorescens* MFN1032 were treated with Anakinra, an IL-1R antagonist. In this model, gut ParaP was also preserved in case of *Pseudomonas* infection (Fig. 3C). As expected, IL-1R deficiency did not alter the IL-1 β and TNF- α levels measured in the PP and ileal biopsies after infection by *P. fluorescens* MFN1032 (Fig. 3D, E). Finally, WT mice were treated with a caspase-1 inhibitor (Z-YVAD-FMK), an enzyme involved in the conversion of pro-IL-1 β into the mature form of IL-1 β . Here too, gut ParaP remained unchanged (Fig. 3C).

These experiments suggested that the gut barrier dysfunction was the result of the stimulation of epithelial cells by IL-1 β produced by macrophagic cells. To further dissect the cross talk between immune and epithelial cells, epithelial Caco-2 cells were cocultivated with THP-1 cells in TSs. Before coculture, THP-1 cells were infected or not with *P. fluorescens* MFN1032 for 6 hours and then treated with gentamycin (to kill the extracellular bacteria). Infected THP-1 cells were then added into the basolateral compartment of the TS, and the ParaP of the Caco-2 monolayers was monitored. FD4 flux across Caco-2 monolayers was significantly higher when epithelial cells were cultivated with infected THP-1 cells than with uninfected cells (Fig. 3F). Anakinra reversed the effect of THP-1 infected cells, when added into the basolateral compartment of the TS. By contrast, a pretreatment by infliximab, an anti-TNF monoclonal antibody, did not significantly affect the ParaP (Fig. 3F). As expected, IL-1 β and TNF- α secretions by THP-1 cells in response to *P. fluorescens* MFN1032 were not affected by the 2 tested inhibitors (see Fig. B, Supplemental Digital Content 3, <http://links.lww.com/IBD/A691>). Altogether, these data indicated that IL-1 β carried the effect of *P. fluorescens* MFN1032 on the epithelial permeability.

P. fluorescens MFN1032 Increases MLCK Expression and Activity, Which in Turn Disrupt the Epithelial Barrier

MLCK has been previously reported to play a central role in the regulation of ParaP in response to IL-1 β and pathogens.^{23,30} We thus investigated its role in case of infection by *P. fluorescens* MFN1032. After infection, the ileal mucosa from WT mice exhibited increased mRNA levels of MLCK (Fig. 4A). In contrast, no increase of MLCK mRNA expression was observed in *IL-1R*^{-/-} mice (Fig. 4A). In vitro, Caco-2 cells cocultured with *P. fluorescens* MFN1032-infected THP-1 cells triggered an increased expression of MLCK protein (Fig. 4B). Treatment with ML-7, a MLCK inhibitor, suppressed the effect of *P. fluorescens* MFN1032 on gut ParaP in vivo (Fig. 4C). Similarly, Caco-2 cells treated with ML-7 failed to exhibit an increased ParaP when cocultured with THP-1 cells infected by *P. fluorescens* MFN1032 (Fig. 4D). As expected, IL-1 β and TNF- α secretions

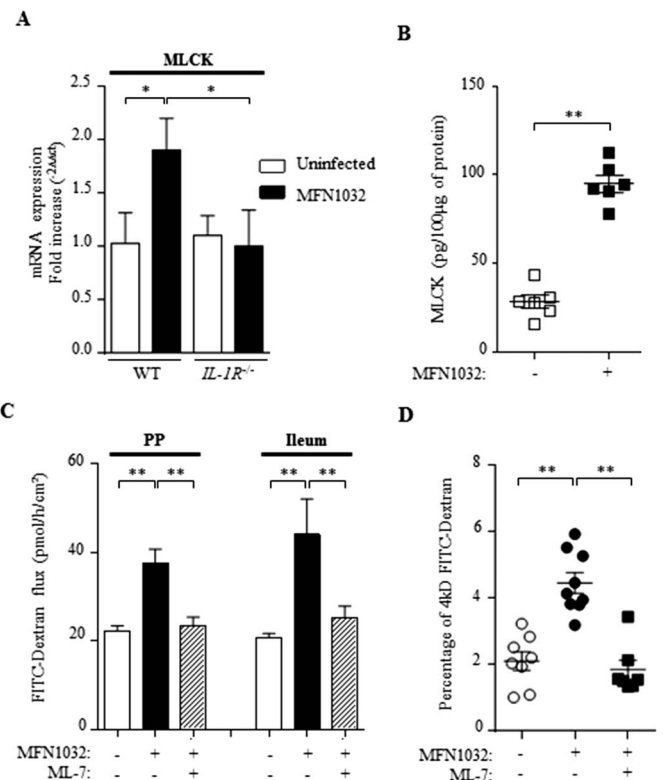


FIGURE 4. *P. fluorescens* MFN1032 increases MLCK expression and activity, which in turn disrupt the epithelial barrier function by a mechanism that is IL-1R-dependent. A, MLCK mRNA expression from ileal mucosa was determined by real-time PCR in WT or *IL-1R*^{-/-} mice previously inoculated with 10⁸ CFU of *P. fluorescens* MFN1032 for 7 days. B, Caco-2 cells were cultivated on TSs. THP-1 cells infected by *P. fluorescens* MFN1032 were then added into the basolateral compartment, and MLCK expression was measured by enzyme-linked immunosorbent assay. C, WT or *IL-1R*^{-/-} mice were intraperitoneally treated with ML-7 (2 mg/kg), an inhibitor of MLCK, for 2 consecutive days before experimentation. Then, biopsies from ileal mucosa and PP were mounted in UC and incubated with *P. fluorescens* MFN1032. ParaP was monitored by measuring the flux of FD4 for 1 hour. D, Caco-2 cells were cultivated on TSs. THP-1 cells infected with *P. fluorescens* MFN1032 were then added into the basolateral compartment, and ParaP was measured. To investigate the involvement of myosin light chain kinase in the increase of ParaP, Caco2 cells were treated for 24 hours with ML-7 (50 μ g/mL). Data are expressed as mean \pm SEM; **P* < 0.05, and ***P* < 0.01; n = 7–9 per group; 3 separate experiments.

by THP-1 cells in response to *P. fluorescens* MFN1032 were not affected by the MLCK inhibitor (see Fig. B, Supplemental Digital Content 3, <http://links.lww.com/IBD/A691>). Together, these results evidenced that *P. fluorescens* strain MFN1032 increases the ParaP by upregulating the activity and expression of MLCK.

IL-1 β Secretion by *P. fluorescens* MFN1032-Infected Macrophages is Nod2 Dependent

Because *NOD2* polymorphisms are characterized by an ileal barrier dysfunction⁴⁵ and the presence of I2 into the serum

is associated with the presence of CD ileal lesions,³⁹ we investigated the role of *Nod2* in case of infection by *P. fluorescens* MFN1032. WT, *Nod2*^{-/-} and mice carrying a mutation homologous to the human 1007 frame shift CD-associated mutation (*Nod2*^{2939iC}) were ig inoculated with 10⁸ CFU of *P. fluorescens* MFN1032 during 7 days. Next, the ParaP was determined by measuring the FD4 level in the serum. As previously shown, increased levels of FD4 were observed in infected WT mice (Fig. 5A). In *Nod2*^{-/-} and *Nod2*^{2939iC} mice, the basal levels of FD4 were high as previously reported.¹⁹ They did not increase after infection by *P. fluorescens* MFN1032 (Fig. 5A). Similar results were obtained by ex vivo measurement of the ParaP through ileal biopsies mounted in UC (Fig. 5B and see Fig. A, Supplemental Digital Content 4, <http://links.lww.com/IBD/A692>). In *Nod2*^{-/-} and *Nod2*^{2939iC} mice, unlike to WT mice, *P. fluorescens* MFN1032 failed to increase the secretions of IL-1 β and TNF- α in the PP and ileum (Fig. 5C).

To confirm the defect of IL-1 β secretion in case of *Nod2* deficiency, BMSC from WT or *Nod2*^{-/-} mice were differentiated into M Φ and infected with the clinical bacterial strain. *P. fluorescens* MFN1032 induced a lower expression of IL-1 β and TNF- α in *Nod2*^{-/-} M Φ (Fig. 5D). To further confirm this finding, *Nod2* was inhibited by RNA silencing in THP-1 cells. We observed a suppression of IL-1 β synthesis after exposure to *P. fluorescens* MFN1032 (Fig. 5E and see Fig. B, Supplemental Digital Content 4, <http://links.lww.com/IBD/A692>). In contrast, TNF- α secretion was not affected by *Nod2* RNA silencing (Fig. 5F). In contrast, TLR-2 and TLR-4 inhibition did not affect IL-1 β or TNF- α synthesis (Fig. 5E, F and see Fig. B, Supplemental Digital Content 4, <http://links.lww.com/IBD/A692>). These results showed that *Nod2* expression is required for the secretion of IL-1 β by macrophages in response to *P. fluorescens* MFN1032.

To further explore the role of *Nod2*, WT and *Nod2*^{2939iC} mice were intraperitoneally inoculated with MDP every 2 days during the infection by *P. fluorescens* MFN1032. PP and ileal biopsies were mounted in UC to measure the ParaP. MDP abolished the increase of ParaP induced by *P. fluorescens* MFN1032 in WT mice (Fig. 6A). In contrast and as expected, no impact of MDP treatment was observed in *Nod2*^{2939iC}-deficient mice infected with *P. fluorescens* MFN1032 (Fig. 6A).

MDP treatment also reduced the secretion of IL-1 β (but not TNF- α) in response to *P. fluorescens* MFN1032 in BMSC differentiated into M Φ or in THP-1 cells infected with *P. fluorescens* MFN1032 (Fig. 6B–D). As expected, when *Nod2* (or its downstream ligand RICK) was inhibited by RNA silencing, the effect of MDP was abrogated in THP-1 cells (Fig. 6D and see Fig. C, Supplemental Digital Content 4, <http://links.lww.com/IBD/A692>). Altogether, these results evidenced that MDP inhibited the production of IL-1 β in response to *P. fluorescens* MFN1032.

Hematopoietic *Nod2* Mediates *P. fluorescens* MFN1032 Epithelial Barrier Dysfunction

Nod2 is expressed in both hematopoietic and nonhematopoietic cells lineages. To determine whether the increased ParaP

depends on *Nod2* expression in epithelial cells or in immune cells, we constructed chimeric mice by bone marrow transplantation. Three months after bone marrow transfer, the expressions of CD45.Ly1 (donors) and CD45.Ly2 (recipients) indicated about 95% efficiency of bone marrow reconstitution for CD3⁺, CD19⁺, and CD11c⁺ cells in both spleen and PP (data not shown). *Nod2* deletion in the hematopoietic compartment was sufficient to increase the ileal ParaP under basal condition (Fig. 7A). After infection by *P. fluorescens* MFN1032, ParaP of the ileal mucosa was increased only when *Nod2* was expressed in the hematopoietic cell lineage (WT \rightarrow WT and WT \rightarrow *Nod2*^{-/-}) (Fig. 7A). Conversely, when *Nod2* was deleted within the hematopoietic compartment (*Nod2*^{-/-} \rightarrow *Nod2*^{-/-} and *Nod2*^{-/-} \rightarrow WT), the gut ParaP was similar regardless of the presence or not of *P. fluorescens* MFN1032 (Fig. 7A).

Chimeric mice were then treated with MDP and infected with *P. fluorescens* MFN1032. *Nod2* stimulation in chimeric mice expressing *Nod2* in their hematopoietic compartment (WT \rightarrow WT and WT \rightarrow *Nod2*^{-/-}) reduced the increase of ParaP (Fig. 7B). We thus concluded that *Nod2* expression in the hematopoietic cell lineages was responsible for the barrier dysfunction after *P. fluorescens* MFN1032 infection.

DISCUSSION

An abnormal host–microbial interaction affecting preferentially the terminal segment of the small bowel is supposed to contribute to ileal CD.⁶ However, the nature of the causative bacteria playing a role in CD remains a subject of debate. While many groups work on the dysbiosis hypothesis (mainly based on the observation that the gut microbiota is different in patients with CD when compared with controls), others explore the role of more specific microbial agents. Among them, *Mycobacterium avium paratuberculosis*, *Escherichia coli*, *Yersinia* species, or *P. fluorescens* (and others) is on the list of proposed candidates. *Yersinia* and *E. coli* LF82 are known to alter the barrier function of the intestinal mucosa.^{23,30,33,46} However, little is known about the impact of *P. fluorescens* on the intestinal barrier.

P. fluorescens is present at low level in the gut, and it has been linked to CD with ileal lesions.^{29,39} Because CD has been related to abnormal host–microbial interactions affecting preferentially the terminal segment of the small bowel at PP location,⁶ we have determined the ability of *P. fluorescens* strains to disrupt the epithelial barrier of the ileal mucosa and PP. For that, we have selected 2 strains of *P. fluorescens*: a classical psychrotrophic strain (MF37) able to grow at low temperatures and a characterized clinical strain (MFN1032) adapted to grow at 37°C. The behavior of these bacteria was compared to the opportunistic pathogen *P. aeruginosa* (PAO1). All the *Pseudomonas* species were able to alter the gut permeability, suggesting that the increased permeability is a general property of *Pseudomonas* colonization in the digestive tract. This result extends the previous report showing that *Pseudomonas* species increase the ParaP of polarized Caco-2/TC7 monolayers.³⁸

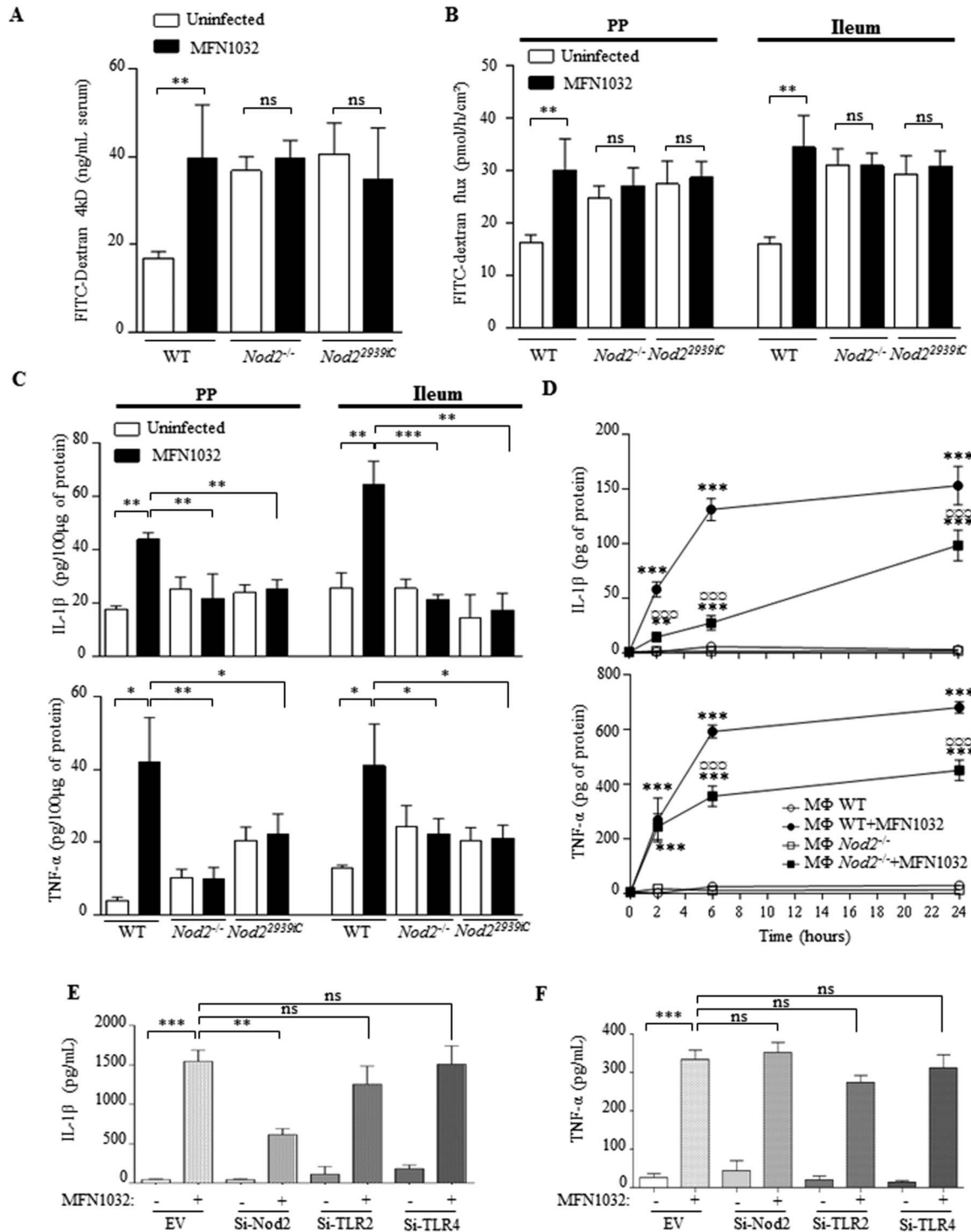


FIGURE 5. Nod2 is required to alter the epithelial barrier function. A–C, WT, *Nod2*^{-/-}, and *Nod2*^{2939C} mice were ig inoculated with 10⁸ CFU of *P. fluorescens* MFN1032 for 7 days. A, ParaP was investigated by measuring the quantity of FD4 in the serum after 6 hours of FD4 oral inoculation. After mice were killed, biopsies from PP or ileum were mounted in UC, and (B) the ParaP was measured together with (C) IL-1β and TNF-α levels (enzyme-linked immunosorbent assay). D, Bone marrow stem cells from WT and *Nod2*^{-/-} mice were differentiated into MΦ and infected with *P. fluorescens* MFN1032 for 2, 6, or 24 hours. Then, IL-1β and TNF-α levels were analyzed by enzyme-linked immunosorbent assay in the supernatant. E and F, THP-1 cells were transfected with siRNA against NOD2, TLR-2, TLR-4, or not targeting siRNA (NT) and infected by *P. fluorescens* MFN1032. After centrifugation, the levels of (E) IL-1β and (F) TNF-α were measured in the supernatant. Data are expressed as mean ± SEM; ****P* < 0.01 and *****P* < 0.001; ns*P* < 0.05 versus MΦ WT + MFN1032; n = 6–14 per group; 3 separate experiments.

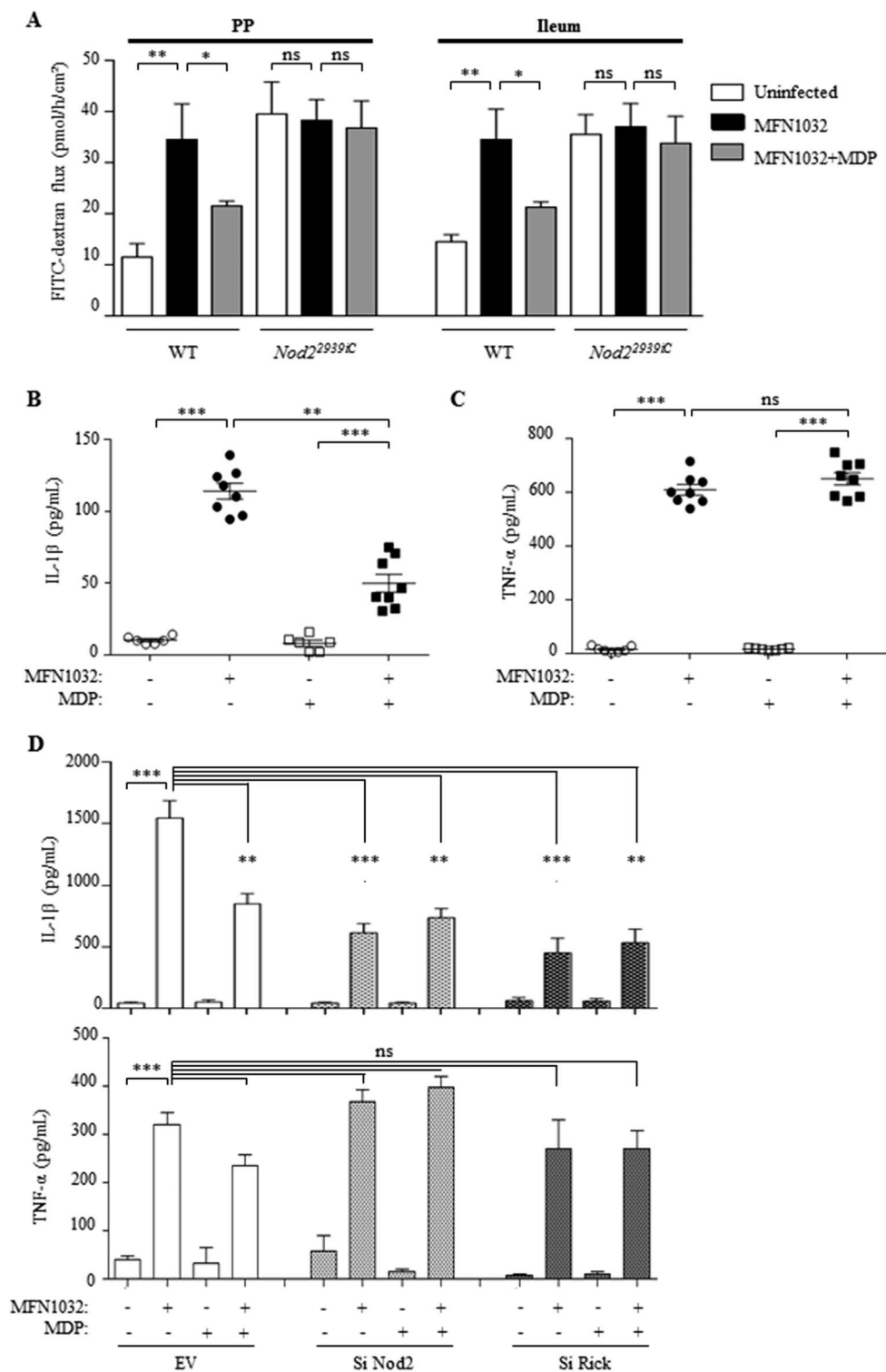
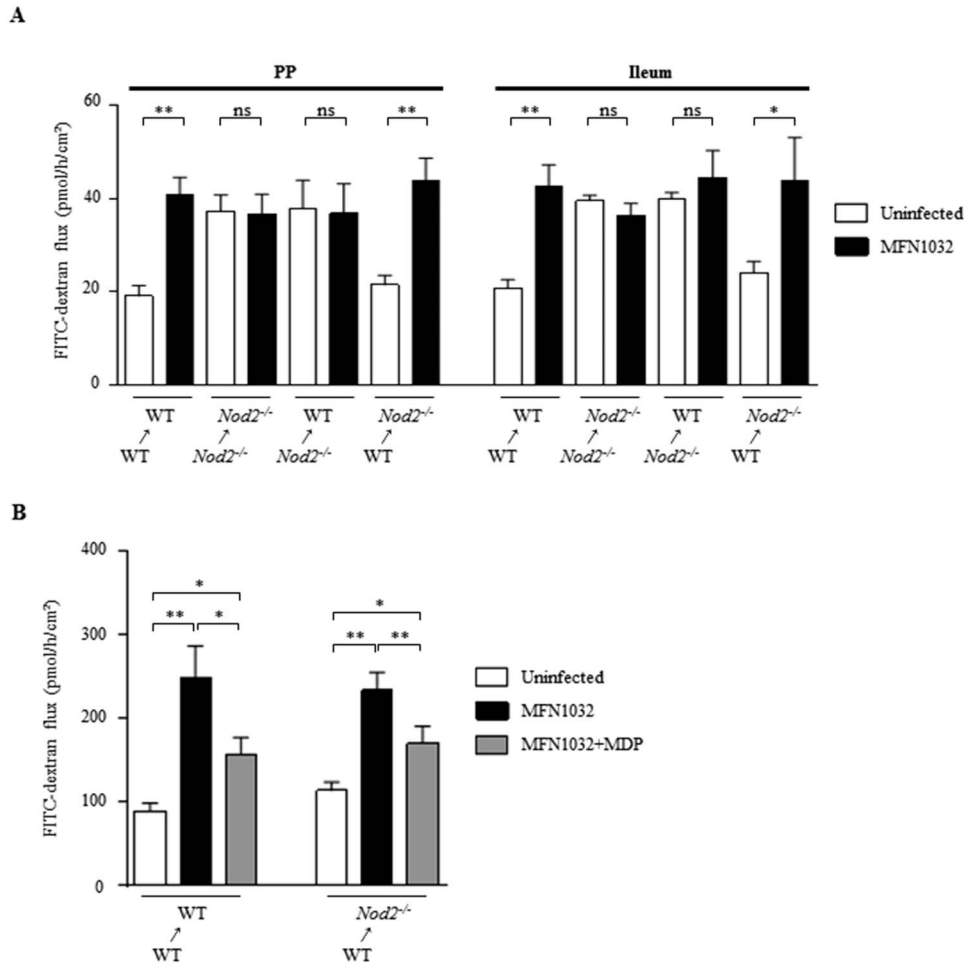


FIGURE 6. MDP treatment abrogates the increase of ParaP and IL-1β synthesis. A, WT and *Nod2*^{2939C} mice were ig inoculated with 10⁸ CFU of *P. fluorescens* MFN1032 for 7 days and treated with MDP (100 μg) every 2 days. After mice were killed, biopsies from PP and ileal mucosa were mounted in UC, and the ParaP was measured for 1 hour. B and C, BMSC from WT mice were differentiated into MΦ cells and treated with MDP (20 μg/mL) 24 hours before infection by *P. fluorescens* MFN1032. After 6 hours, IL-1β (B) and TNF-α (C) levels in the supernatant were analyzed by enzyme-linked immunosorbent assay. D, THP-1 cells were transfected with siRNA against NOD2 or Rick or NT siRNA and treated with MDP (20 μg/mL) 24 hours before infection by *P. fluorescens* MFN1032. After 6 hours, the levels of IL-1β and TNF-α were measured in the supernatant. Data are expressed as mean ± SEM; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; n = 6–14 per group; 3 separate experiments.



However, the different strains used in this study did not exhibit the same amplitude of barrier dysfunction. Unsurprisingly, *P. aeruginosa* PAO1 had the higher impact, whereas *P. fluorescens* MF37 (a nonpathogenic strain) had the less marked effect. It was outside the scope of this study to explore the virulence factors explaining these differences. However, we can note that some differences have yet been reported between these strains. For example, *P. fluorescens* MF37 and *P. fluorescens* MFN1032 displayed similar adhesion potential to Caco-2/TC7, but *P. fluorescens* MFN1032 exhibits a higher cytotoxic effect.³⁷

Several proinflammatory cytokines including IL-1β, TNF-α, and interferon γ are known to alter the gut barrier.^{44,47} Dissecting the mechanisms of the gut barrier dysfunction during *Pseudomonas* infection, we found that it was mainly related to the production of IL-1β. This cytokine is known to increase the gut ParaP in mice^{23,30} and in cell culture models.⁴⁷ It has also been proposed to contribute to the intestinal barrier defect in patients with CD.⁴⁸

As a consequence, IL-1β seems to play a dual role. On one hand, it helps to control the infection by participating in the host inflammatory response. On the other hand, it allows the entry of the luminal content, which may favor a vicious circle and an uncontrolled inflammation.⁴⁹

The mechanism by which IL-1β increases the gut ParaP is the upregulation of both the expression and the activity of epithelial MLCK.⁵⁰ In mouse, IL-1β increases the MLCK mRNA expression in epithelial cells by a mechanism depending on the activation and nuclear translocation of the NF-κB p65 subunit.⁵⁰ In vitro studies based on polarized Caco-2 monolayers suggest that the effect of IL-1β on MLCK expression is mediated by the activation of extracellular signal-regulated kinases 1/2 pathway.⁵¹ The activation of extracellular signal-regulated kinases 1/2 triggers nuclear translocation of Elk-1, which is able to bind a cis-binding motif on the MLCK promoter, promoting MLCK transcription.^{51,52} Noteworthy, *P. aeruginosa* PAO1 is able to stimulate NF-κB activity,

but it has no effect on the activator protein 1 pathway. In contrast, *P. fluorescens* MFN1032 stimulates the activator protein 1 pathway but does not enhance the NF- κ B pathway.^{37,38} Thus, additional studies are requested to further dissect the signaling pathway of MLCK stimulation in the epithelial cells.

Whatever be the signaling cascade involved in the regulation of MLCK expression, the inhibition of MLCK activity abrogates the effect of *Pseudomonas* on the permeability and more generally on IL-1 β -mediated gut dysfunction.⁵⁰ In CD, the pathophysiologic importance of MLCK is supported by the observation that MLCK expression and activity are increased in enterocytes of biopsied patients.⁵³ The impact of MLCK on gut permeability has been studied previously.⁵⁴ Activated MLCK increases the width of epithelial tight junctions, a phenotype that is also reported in CD.⁵⁵

As shown here, the capacity of numerous pathogens to disrupt the epithelial barrier function facilitates not only its own translocation within the gut mucosa but also the passage of the luminal content within the internal milieu. One of the major features of CD pathophysiology is an excess of both ParaP and bacterial translocation.^{2,21} As a result, an overreactivity against several antigens present in the gut lumen (including against *P. fluorescens*) is a key finding in CD.^{29,39}

NOD2 mutations, including a 1007 frame shift mutation, are frequently encountered in patients with ileal CD.⁴⁵ Furthermore, several results indicate that *NOD2* is directly linked to the regulation of IL-1 β synthesis.^{56,57} We thus questioned the relationship between *P. fluorescens* and *NOD2* deficiency. In both *Nod2* knockout mice and mice carrying a mutation homologous to the human 1007 frame shift mutation, *P. fluorescens* was no more able to alter the gut barrier. The presence of functional *Nod2* in the hematopoietic compartment is required for *P. fluorescens* MFN1032 to increase the ParaP by IL-1 β secretion. RICK (adownstream partner of *Nod2*) was also involved in the synthesis of IL-1 β .²³ In contrast, TLR-2 and TLR-4 were not involved in the IL-1 β synthesis in response to *P. fluorescens* MFN1032. Finally, IL-1 β production may be prevented by MDP, a *Nod2* ligand.

A similar mechanism has been reported for *Y. pseudotuberculosis*.²³ In that case, the acetylation of RICK (a *Nod2* downstream effector) by the *Yersinia* virulence factor YopJ reduces its affinity for *NOD2*.²³ This effect is reversible by MDP pretreatment, suggesting that it is a dynamic process. When RICK is inhibited, *NOD2* is able to form a complex with caspase 1 and consequently to produce mature IL-1 β . In this model, *NOD2* is in equilibrium with its 2 partners: RICK and caspase 1. Under MDP activation, *NOD2*/RICK interaction is favored, resulting in NF- κ B and MKK signaling while in case of bacterial infection by *Y. pseudotuberculosis*, *Nod2* is free to activate caspase 1, resulting in IL-1 β secretion. All the data presented here suggest that a similar model may be proposed for *P. fluorescens*. Interestingly, *Salmonella* species also take advantage of IL-1 β secretion and of a low grade of mucosal inflammation.⁵⁸ This effect is mediated by the effector SopE, which activates caspase-1 and IL-1 β synthesis.⁵⁹

The role of *P. fluorescens* in CD has been initially supported by the presence of antibodies directed against the I2

sequence, which is specific to the bacteria.^{29,39} The cold chain hypothesis also points out *P. fluorescens* as a candidate bacteria for CD because it is able to grow at low temperatures.²⁵ According to this hypothesis, refrigerated food may carry bacteria (like *Pseudomonas* or *Yersinia* species), which triggers the inflammation in genetically at-risk patients. In the present state of our knowledge, the absence of functional *Nod2* in the host is characterized by a gut barrier dysfunction at the basal level.^{19,22} We show here that in case of infection by *P. fluorescens*, MDP stimulation is not able to restore the barrier function in case of *Nod2* mutation. As a result, the antigenic load of the mucosa is increased and may result in a chronic inflammation.

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-Article 6-

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ORIGINAL PAPER

***Pseudomonas fluorescens* can induce and divert the human β -defensin-2 secretion in intestinal epithelial cells to enhance its virulence**

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Pseudomonas fluorescens can induce and divert the human β -defensin-2 secretion in intestinal epithelial cells to enhance its virulence

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Abstract The effect of intestinal molecules produced by the host on the virulence of *Pseudomonas fluorescens* is poorly documented. In the present work, we evaluated the secretion of human β -defensin-2 (hBD-2) by enterocytes after infection with *P. fluorescens* (a species previously suggested to be involved in inflammatory bowel disease) and investigated the effect of this host-defense peptide on the bacterial virulence. The results showed that *P. fluorescens* can induce hBD-2 production in Caco-2/TC7 cells via P38 and ERK MAPK-dependent pathways. Surprisingly, the exposure of *P. fluorescens* to low doses of the antimicrobial peptide was found to enhance its cytotoxic and proinflammatory effects suggesting a potential feedback mechanism in the dialog between bacteria and the host.

Keywords *P. fluorescens* · Caco-2/TC7 · hBD-2 · NF- κ B · MAPK · Virulence

Introduction

The intestinal epithelium contains a great diversity of cell types, including disseminated neuroendocrine and defense

cells. These cells are the source of a multitude of signal molecules and antibacterial substances. Among these factors are defensins, inducible or constitutive antimicrobial peptides, expressed by various cell types (Selsted and Ouellette 2005; Nuding et al. 2009). These small (3–5 kDa) cationic peptides are classified as α -, β -, or θ -defensins, on the basis of the position of their intramolecular disulfide bridges. An alteration of defensin production should participate in the pathogenesis of inflammatory bowel disease (IBD). In CD, reduced α -defensin levels have been seen in patients with ileal inflammation, whereas reduced β -defensin levels were measured in those with colonic involvement (Ramasundara et al. 2009; Chamailard and Dessein 2011). An attenuated expression of defensins, such as human β -defensin-2 (hBD-2), could compromise host immunity and alter the balance between protection from pathogens and tolerance to the normal flora (Ramasundara et al. 2009). hBD-2 is an antimicrobial peptide having a broad antibiotic spectrum against gram-negative and gram-positive bacteria, as well as against fungi and viruses (Ganz 2005). It is induced by cytokines or in response to inflammatory stimuli such as bacterial infections (Yoon et al. 2010). Activation of AP-1 (activator protein 1) and/or NF- κ B (nuclear factor κ B) transcription factors via the MAPK (mitogen-activated protein kinases) including P38, JNK (c-jun N-terminal kinase), and ERK1/2 (extracellular signal-regulated protein kinases), via the MAPK/I- κ B kinase (IKK)/NF- κ B, or via JNK/NF- κ B pathways are known to be important for the induction of defensins and proinflammatory factors in several cell lines (Wehkamp et al. 2004; Schneider et al. 2005; Wehkamp et al. 2006; Yoon et al. 2010; Jung et al. 2012).

Unlike *P. aeruginosa* which is largely described as an opportunistic pathogen (Okuda et al. 2010), the virulence of *P. fluorescens* has often been underestimated. This bacterium

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is generally considered as a psychrotrophic microorganism, but some clinical strains such as *P. fluorescens* MFN1032 are adapted to the human physiological temperature (Chapalain et al. 2008), show an important pathogenic potential (Sperandio et al. 2010), and trigger a specific inflammatory response in enterocytes (Madi et al. 2010a). The adaptation of *P. fluorescens* to higher temperatures can lead to strains of increased virulence, almost similar to *P. aeruginosa* (Picot et al. 2004; Chapalain et al. 2008; Donnarumma et al. 2010). Interestingly, a highly specific antigen of *P. fluorescens*, called I2, is detected in the serum of patients with Crohn's disease (CD), and a direct link between the severity of CD and the level of circulating I2 has been demonstrated (Dalwadi et al. 2001). Moreover, we found recently that *P. fluorescens* can be cytotoxic in Caco-2/TC7 intestinal cells, invade the target cell, and translocate from the luminal to the internal compartment (Madi et al. 2010b).

A dysregulation of defensin production may occur in CD, altering the antimicrobial activity of the epithelium (Wehkamp et al. 2005), but the effect of the intestinal molecules produced by the host, including hBD-2, on the virulence of *P. fluorescens* is completely unknown. Therefore, the aim of our study was to evaluate the induction of hBD-2 secretion by enterocytes after infection with *P. fluorescens* (a species previously suggested to be involved in CD) in comparison with the opportunistic pathogen *P. aeruginosa* PAO1. We also investigated the effect of this host-defense peptide on the bacterial virulence.

Materials and methods

Bacterial strains

Pseudomonas aeruginosa PAO1 is a common laboratory reference strain (Klockgether et al. 2010). *P. fluorescens* MF37 is a rifampicin-resistant natural mutant of the strain MF0 (biovar V), originally identified in crude milk (Burini et al. 1994). *P. fluorescens* MFN1032 is a clinical biovar I strain collected in a hospital of Haute-Normandie (France) (Chapalain et al. 2008). *P. aeruginosa* PAO1 and the two strains of *P. fluorescens* were cultivated in ordinary nutrient broth (Merck) at 37 and 28 °C, respectively.

Cell culture

The human colon adenocarcinoma cell line Caco-2/TC7 was used between passages 10 and 35 to avoid derivation of the cells. Caco-2/TC7 cells were grown at 37 °C with 5 % CO₂, in Dulbecco's modified Eagle's medium (Sigma) containing 20 % fetal calf serum (FCS) supplemented with 2 mM of L-glutamine, 100 U/ml each of penicillin and streptomycin and 1 % non-essential amino acids.

For the experimental assays, the cells were cultivated in 24-well plates until confluence.

Bacterial treatment with hBD-2 and infection of Caco-2/TC7 cells

At the beginning of the exponential growth phase, 1 µg/ml of hBD-2 was added to the bacterial cultures. After 6 h of exposition to the peptide, the bacteria were centrifuged for 5 min at 8,000g and resuspended in cell culture medium without antibiotics and serum. Confluent Caco-2/TC7 monolayers were then infected with the hBD-2-treated bacteria at a MOI (multiplicity of infection) of 100:1 and incubated overnight at 37 °C in 5 % CO₂.

Cytotoxicity assay

Caco-2/TC7 cell death was determined at the end of the infection period (15 h) by measurement of lactate dehydrogenase (LDH) release using the Cytotox 96[®] enzymatic assay (Promega) as previously described (Madi et al. 2010a). LDH release by Caco-2/TC7 cells exposed to Triton X100 (0.9 %) was used as a control of total cell lysis (100 %). The background level (0 % LDH release) was determined in the medium of uninfected cells. The percentage of cytotoxicity was then calculated following the manufacturer's instructions.

Adhesion assay

Bacterial adhesion to confluent Caco-2/TC7 cells was quantified after 4 h of infection. Monolayers were washed with PBS to remove non-adherent bacteria and lysed by incubation for 15 min with 0.1 % Triton X100. The lysates were then diluted and plated onto ordinary nutrient agar to determine the number of cell-associated (adherent and invasive) bacteria.

Quantification of IL-8 and β-defensin-2

The level of IL-8 produced by Caco-2/TC7 cells was measured in the supernatant at the end of infection using an ELISA Quantikine kit (R&D Systems) according to the supplier's protocol. β-defensin-2 was quantified with the Defensin 2, beta (Human)–ELISA Kit (Phoenix Pharmaceuticals inc).

Pharmacological inhibition of the MAPK and NF-κB pathways

In order to analyze the involvement of the MAPK and NF-κB pathways in hBD-2 induction by *Pseudomonas*, we used specific inhibitors of the MAPK or NF-κB cascades.

The three activation routes of the MAPK in Caco-2/TC7 cells were blocked by use of the p38 MAPK inhibitor SB203580 (Sigma), the JNK inhibitor SP600125 (Sigma), or the ERK1/2 inhibitor U126 monoethanolate (Sigma) (Yoon et al. 2010). The NF- κ B pathway was blocked using the NF- κ B activation inhibitor NAI (Calbiochem). These pharmacological agents were added in the culture cell medium 1 h prior to the bacterial infection, at a final concentration of 50 μ M for SB203580 and SP600125 or 10 μ M for U126 monoethanolate and NAI.

Statistical analysis

All experiments were conducted independently at least three times. The results are expressed as mean \pm standard error (SE). Analysis of statistical significance was performed by ANOVA with Student's *t* test.

Results

Pseudomonas strains induce hBD-2 secretion by Caco-2/TC7

As shown in Fig. 1, a spontaneous low basal secretion of hBD-2 (4.6 \pm 0.6 pg/ml) was found for uninfected Caco-2/TC7 cells. The three strains of *Pseudomonas* studied induced a marked increase in hBD-2 production. However, the results obtained were unexpected. Surprisingly, the higher production of hBD-2 secretion was observed in the presence of the psychrotrophic strain *P. fluorescens* MF37 (32.2 \pm 1.2 pg/ml). The clinical strain of *P. fluorescens* MFN1032 and the opportunistic pathogen *P. aeruginosa* PAO1 provoked a lower increase in hBD-2 reaching 16.9 \pm 5.5 and 16.5 \pm 1.4 pg/ml, respectively.

The MAPK and NF- κ B pathways are involved in hBD-2 induction by *Pseudomonas*

In order to identify the mechanisms involved in hBD-2 stimulation by *P. fluorescens* and *P. aeruginosa*, Caco-2/

TC7 cells were pretreated with different inhibitors of the MAPK or NF- κ B pathways before the infection by the bacteria. The results showed that a treatment of Caco-2/TC7 cells with SB203580, a specific inhibitor of the P38-dependent MAPK activation route, strongly reduced the hBD-2 secretion induced by *P. fluorescens* MF37 (Fig. 2a). The inhibitor of MAPK/ERK1/2 pathway, U126 monoethanolate, had the same effect as SB203580. In contrast, the inhibitor of MAPK/JNK pathway, SP600125, did not modify the hBD-2 production induced by *P. fluorescens* MF37. The NF- κ B activation inhibitor NAI also had no effect on the level of hBD-2 produced after infection by *P. fluorescens* MF37. Experiments realized using *P. fluorescens* MFN1032 led to almost similar results, but a smaller reduction in hBD-2 secretion was obtained with the inhibitors SB203580 and U126 compared to *P. fluorescens* MF37 (Fig. 2b). By contrast, the results were markedly different for *P. aeruginosa* PAO1 (Fig. 2c). The JNK inhibitor of MAPK, SP600125, and the NF- κ B activation inhibitor NAI both reduced the induction of hBD-2 secretion in Caco-2/TC7 whereas the inhibitor of the ERK1/2 pathway, U126 monoethanolate, was inactive.

hBD-2 increases the cytotoxic effect of *P. fluorescens* MF37

We observed that *P. fluorescens* and *P. aeruginosa* modulate hBD-2 secretion by Caco-2/TC7 cells through different specific activation pathways, but it has remained unclear whether this peptide can be sensed by the bacteria and influence their virulence. Thus, to start exploring this aspect, cytotoxicity test was performed on Caco-2/TC7 cells using bacteria pretreated with hBD-2 (1 μ g/ml). This concentration of peptide was selected in a preliminary experiment to have no effect on the bacterial growth and survival (data were not shown). The results of cytotoxicity assay are presented in Fig. 3. We found that a pretreatment of *P. fluorescens* MF37 with hBD-2 (1 μ g/ml) resulted in a significant increase in its cytotoxicity toward Caco-2/TC7 cells. The cytotoxic effect of *P. fluorescens* MFN1032 was

Fig. 1 Secretion of hBD-2 in Caco-2/TC7 cells after infection with *P. fluorescens* MF37, *P. fluorescens* MFN1032, and *P. aeruginosa* PAO1. **P* < 0.05, ****P* < 0.001, versus uninfected cells

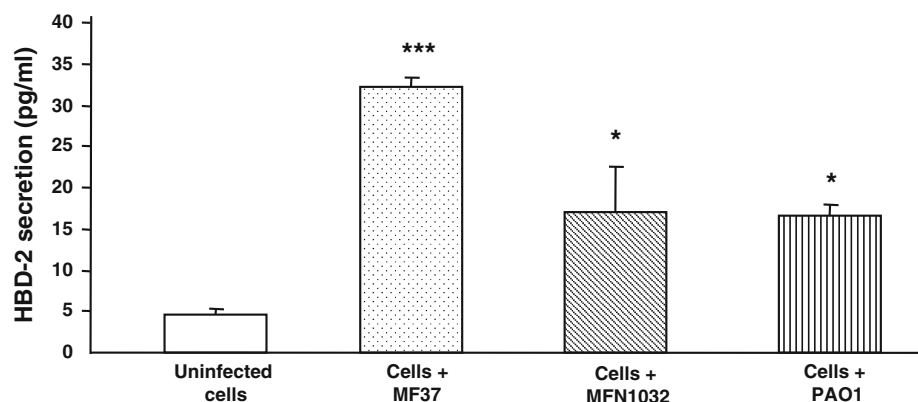


Fig. 2 Effect of p38 MAPK inhibitor (SB203580), JNK inhibitor (SP600125), ERK1/2 inhibitor (U126), and NF- κ B activation inhibitor (NAI) on the secretion of hBD-2 by Caco-2/TC7 cells exposed to *P. fluorescens* MF37 (a), *P. fluorescens* MFN1032 (b), and *P. aeruginosa* PAO1 (c). ns not significant, * $P < 0.05$, *** $P < 0.001$, versus infected cells without inhibitor

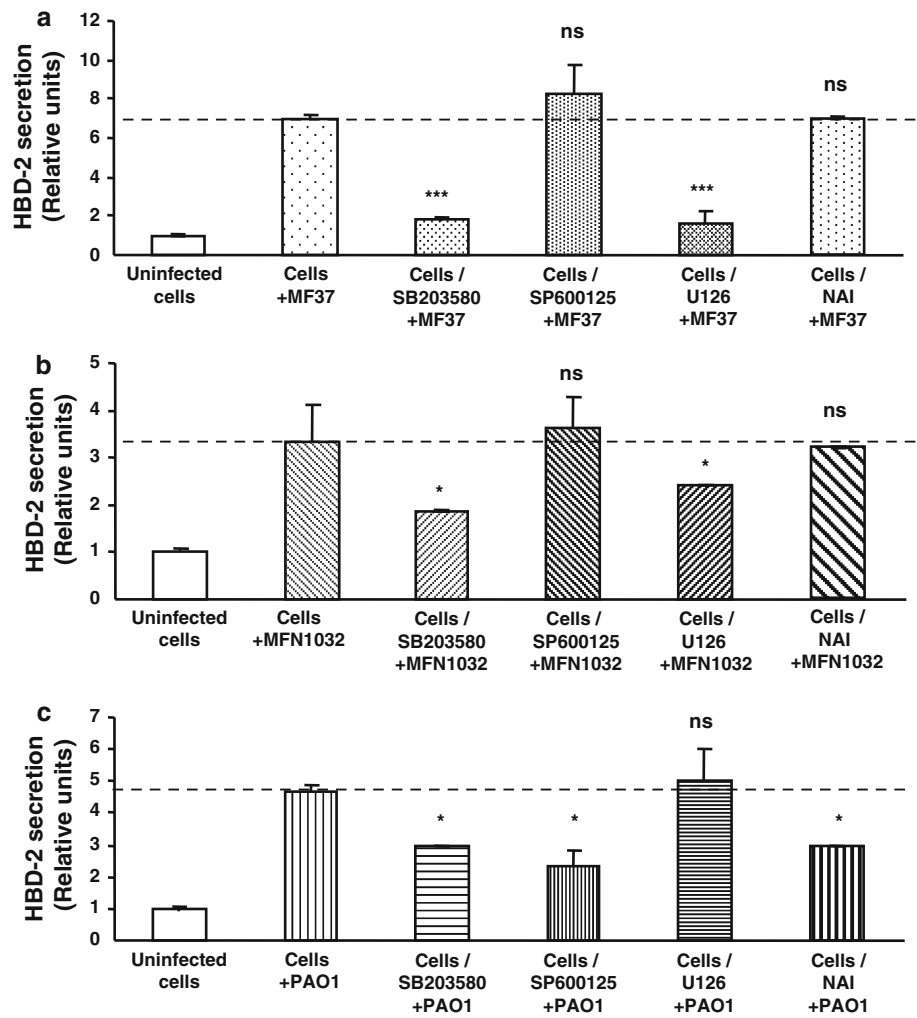
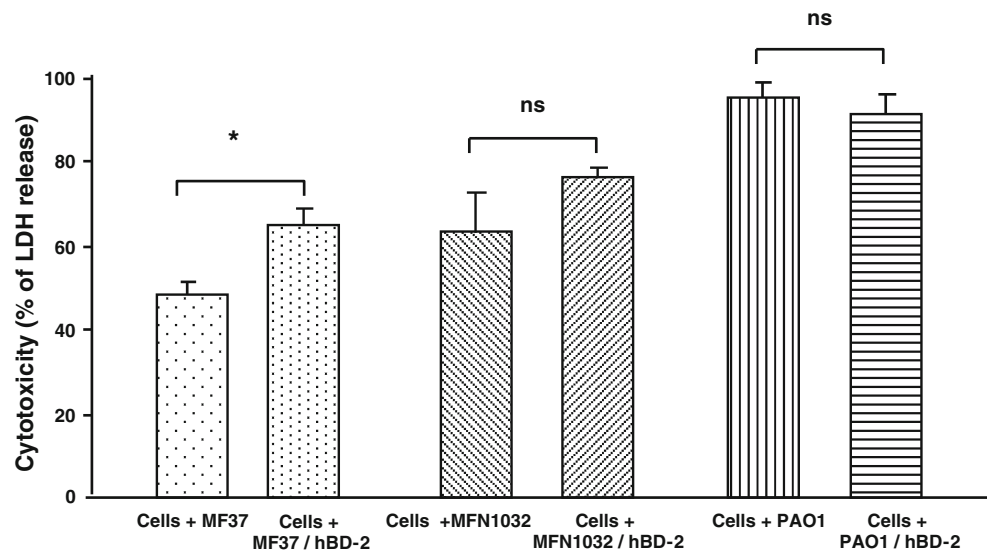


Fig. 3 Effect of hBD-2 exposure (1 μ g/ml) on *P. fluorescens* MF37, *P. fluorescens* MFN1032, and *P. aeruginosa* PAO1 cytotoxic activity toward Caco-2/TC7 cells. Cytotoxicity was evaluated by measurement of lactate dehydrogenase (LDH) released from damaged cells. ns not significant, * $P < 0.05$, versus cells infected by untreated bacteria



also promoted by hBD-2, but the observed increase was insignificant. Finally, no apparent effect was seen for *P. aeruginosa* PAO1.

hBD-2 modulates the proinflammatory effect of *Pseudomonas*

To further explore the modulation of bacterial virulence by hBD-2, the proinflammatory cytokine IL-8 secreted by Caco-2/TC7 was quantified (Fig. 4). We found that a pretreatment of *P. fluorescens* MF37 with hBD-2 (1 µg/ml) provoked a marked increase in its capacity to stimulate IL-8 secretion by Caco-2/TC7 cells. By contrast, we observed a decrease in the IL-8 secreted by Caco-2/TC7 after infection with *P. fluorescens* MFN1032 when the strain was pretreated with hBD-2 (1 µg/ml). Finally, the pretreatment of *P. aeruginosa* PAO1 with hBD-2 (1 µg/ml) increased its proinflammatory potential, but this effect was weaker than for *P. fluorescens* MF37.

hBD-2 affects the adhesion potential of *P. fluorescens* MF37 and *P. aeruginosa* PAO1

Since bacterial adhesion could play a role in virulence, we then investigated the effect of hBD-2 on *Pseudomonas* adhesion to Caco-2/TC7 cells (Fig. 5). We found that the adhesion of *P. fluorescens* MF37 significantly increased (1.4 fold increase) when the bacteria were pretreated with hBD-2 (1 µg/ml). By contrast, the binding index of *P. fluorescens* MFN1032 exposed to hBD-2 (1 µg/ml) remained unchanged and that of *P. aeruginosa* PAO1 was clearly reduced (1.3 fold decrease), suggesting that hBD-2 triggered different adaptative responses in the three strains of *Pseudomonas*.

Discussion

The three *Pseudomonas* strains studied were found to be able to induce hBD-2 secretion in Caco-2/TC7. However, the psychrotrophic strain *P. fluorescens* MF37, logically considered as the less virulent, was responsible for the strongest hBD-2 induction, whereas the clinical strain *P. fluorescens* MFN1032 and the opportunistic pathogen *P. aeruginosa* PAO1 provoked only a moderate stimulation of hBD-2. We can hypothesize that the host may be adapted to the encounter of the opportunistic pathogen *P. aeruginosa* PAO1 or the clinical *P. fluorescens* strains such as MFN1032. Moreover, a recent study showed that *P. aeruginosa* is able to subvert the host innate immune response through manipulation of hBD-2 expression (Dössel et al. 2012). On the contrary, infection with an unknown bacterium may provoke a stronger stimulation of the immune system. The use of pharmacological inhibitors showed that the three bacterial strains induced hBD-2 by different pathways. In agreement with our previous data (Madi et al. 2010a), hBD-2 induction by *P. fluorescens* appeared to be mediated through the AP-1 pathway, activating P38 MAPK and/or ERK1/2, and was independent of NF-κB nuclear factor. Conversely, the effect of *P. aeruginosa* PAO1 on hBD-2 secretion was inhibited by blockers of the JNK, P38, or NF-κB pathways, indicating that this strain acted through both MAPK and NF-κB activation routes. This result is different from that obtained previously using a gene reporter strategy showing that *P. aeruginosa* was not acting on Caco-2/TC7 cells through the AP-1 nuclear factor (Madi et al. 2010a). However, cross talks exist between the different pathways and *P. aeruginosa* may activate a signaling cascade involving both P38/IKK/NF-κB and/or JNK/NF-κB as observed with the

Fig. 4 Effect of hBD-2 exposure (1 µg/ml) on the modulation of the proinflammatory potential of *P. fluorescens* MF37, *P. fluorescens* MFN1032, and *P. aeruginosa* PAO1: activation of interleukin-8 (IL-8) secretion by Caco-2/TC7. * $P < 0.05$, *** $P < 0.001$, versus cells infected with untreated bacteria

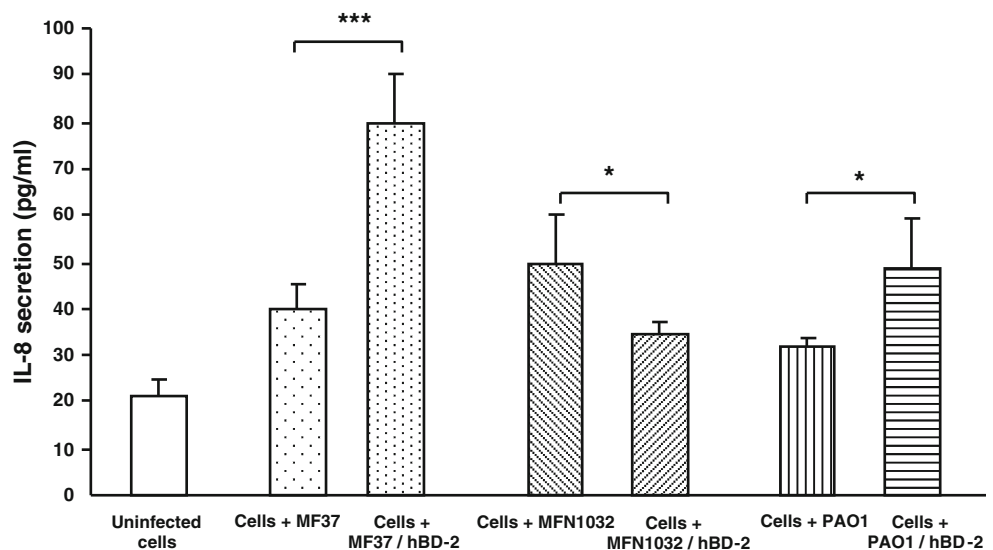
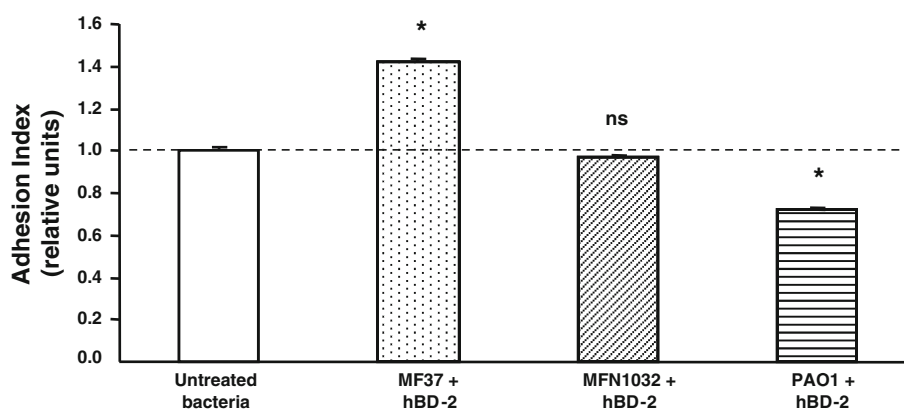


Fig. 5 Effect of hBD-2 exposure (1 $\mu\text{g/ml}$) on the adhesion potential of *P. fluorescens* MF37, *P. fluorescens* MFN1032, and *P. aeruginosa* PAO1 on Caco-2/TC7 cells. Results are expressed as relative units compared to the untreated bacteria of the same strain as control. *ns* not significant, * $P < 0.05$, versus hBD-2-untreated bacteria



enterotoxin of *Bacteroides fragilis* (Yoon et al. 2010) and in the inflammatory response of dendritic cells (Jung et al. 2012).

Pseudomonas are highly resistant microorganisms, and several reports indicate that the minimum bactericidal concentration (MBC) of hBD-2 on these bacteria vary from 10 to more than 250 $\mu\text{g/ml}$ (Jackson and Phillips 1996; Huang et al. 2007; Veldhuizen et al. 2008). However, it has been demonstrated that antibacterial peptides can affect the physiology of microorganisms at concentrations well below those having any effect on bacterial growth (Hancock and Scott 2000). In a preliminary study, *P. fluorescens* MF37, *P. fluorescens* MN1032, and *P. aeruginosa* PAO1 were exposed to increasing concentrations of hBD-2. In order to stay within physiologically relevant conditions, a concentration of 1 $\mu\text{g/ml}$ hBD-2, without effect on the bacterial growth kinetic, was selected for our study. Surprisingly, we observed that this concentration of hBD-2 increased the cytotoxicity and proinflammatory potential of *P. fluorescens* MF37, whereas the effect on *P. aeruginosa* PAO1 and *P. fluorescens* MFN1032 virulence and IL-8 production was marginal or null. This observation could be explained by the fact that unlike *P. fluorescens* MF37, the clinical strain *P. fluorescens* MFN1032 or of course the mesophile bacterium *P. aeruginosa* is adapted to the human physiological environment, so their production of virulence factors may already be maximal because of this adaptation.

The modulation of *P. fluorescens* virulence is sometimes correlated with changes in its lipopolysaccharide (LPS) structure (Picot et al. 2004; Veron et al. 2008). Since LPS is essential in bacterial surface polarity and interactions with eukaryotic cells, we studied the effect of hBD-2 on the adhesion of *Pseudomonas* to Caco-2/TC7 cells. As we could expect, the binding index of hBD-2-treated *P. fluorescens* MF37 showed a marked increase. This should be essential for its cytotoxic and proinflammatory activities.

Conversely, the absence of effect of hBD-2 on the adhesion of *P. fluorescens* MFN1032 is in agreement with the fact that virulence was not increased in this clinical strain and the proinflammatory activity was reduced. The response of *P. aeruginosa* to hBD-2, characterized by a 28 % reduction in adhesion to Caco-2/TC7 cells, was consistent with the absence of modulation of the cytotoxic activity of the bacterium. However, hBD-2-treated *P. aeruginosa* also showed a higher proinflammatory effect with a significant increase in IL-8 secretion. The virulence of this species is associated with the secretion of a multitude of diffusible factors able to act independently of surface contact, and as observed recently, in this species the secretion of virulence factors can be simultaneously up- and down-regulated in the response to a same environmental signal (Blier et al. 2011). In fact, this raise of IL-8 secretion associated with an absence of increased virulence and a lower binding activity to the target cell suggests that hBD-2 may facilitate *P. aeruginosa* to escape from the host recognition system as suggested previously (Bardoel et al. 2011; Dössel et al. 2012).

Finally, our study illustrates the complexity of host-pathogen interaction. We observe that all the *Pseudomonas* strains studied promote hBD-2 secretion but through different mechanisms. The consequences of hBD-2 induction are also very different, depending on the bacteria tested, ranging from a net increase in virulence to a potential escape from the host immune response. In a context where antimicrobial peptides are suggested in replacement to classical antibiotics, this work demonstrates that the bacterial response to these peptides is more complex than expected and will need further investigation to ensure the absence of future emerging resistant strains.

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ORIGINAL PAPER

The pathogenic potential of *Pseudomonas fluorescens* MFN1032 on enterocytes can be modulated by serotonin, substance P and epinephrine

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The pathogenic potential of *Pseudomonas fluorescens* MFN1032 on enterocytes can be modulated by serotonin, substance P and epinephrine

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Abstract *Pseudomonas fluorescens* is a commensal bacterium present at low level in the human digestive tract that has also been reported in many clinical samples (blood, urinary tract, skin, lung, etc.) and sometimes associated with acute opportunistic infections. It has recently been found that the human β -defensin-2 can enhance the pathogenic potential of *P. fluorescens*. In this study, we evaluated the effect of other intestinal molecules (5HT, SP and Epi) on growth and virulence of the clinical strain *P. fluorescens* MFN1032. We found that *P. fluorescens* MFN1032 growth was not mainly affected by these factors, but several modifications in the virulence behavior of this bacterium were observed. 5HT, SP and Epi were able to modulate the motility of *P. fluorescens* MFN1032. 5HT and SP had an effect on pyoverdinin production and IL-8 secretion,

respectively. Infection of Caco-2/TC7 cells with *P. fluorescens* MFN1032 pretreated by SP or Epi enhanced the permeability of the monolayers and led to a partial delocalization of F-actin to the cytoplasm. These findings show that some intestinal molecules can modulate the pathogenic potential of *P. fluorescens* MFN1032. We can hypothesize that this dialogue between the host and the human gut microbiota may participate in health and disease.

Keywords Serotonin · Substance P · Epinephrine · *Pseudomonas fluorescens* · Virulence

Introduction

Pseudomonas is a large genus, currently comprised of more than 100 species with remarkable metabolic and physiologic versatility, enabling them to adapt and colonize diverse ecological niches (Silby et al. 2011). The two best characterized *Pseudomonas* species are *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. *P. aeruginosa* is one of the most common opportunistic pathogens that are present at clinically undetectable levels in the normal gastrointestinal tract (Alverdy et al. 2000). *P. fluorescens* is often considered as a plant-growth-promoting rhizobacterium (Alsohim et al. 2014), but is also present in the human gut as a low level commensal (Wei et al. 2002). Interestingly, a highly specific antigen of *P. fluorescens*, designated as I2, was detected in the serum of 54 % of the patients suffering from Crohn's disease (Sutton et al. 2000), and a correlation between the severity of the pathology and the level of the circulating I2 antigen has been demonstrated (Dalwadi et al. 2001). Recently, a *P. fluorescens*-like bacterium has been isolated from the human stomach (Patel et al. 2013) and the authors estimated that *P. fluorescens* may be as common as *Helicobacter pylori* in the

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gastrointestinal tract. The presence of *Pseudomonas* spp. in 80 % of antral biopsies has already been reported since 1975 (Steer and Colin-Jones 1975), and it has been suggested later that *Pseudomonas* strains may interfere with the identification of *H. pylori* (Domínguez-Bello et al. 2000). In the last few years, the isolation of *P. fluorescens* as the causative agent of nosocomial infections has rapidly increased (Donnarumma et al. 2010; Scales et al. 2014). This bacterium is also commonly isolated from respiratory specimens following lung transplantation (Dickson et al. 2014). Some clinical strains of *P. fluorescens* such as MFN1032 are adapted to the human physiological temperature (Chapalain et al. 2008), show various pathogenic potential (Rossignol et al. 2008, 2009; Sperandio et al. 2010, 2012) and trigger a specific inflammatory response in enterocytes (Madi et al. 2010). In fact, we also found recently that *P. fluorescens* could alter the intestinal barrier function by modulating IL-1 β expression through hematopoietic NOD2 signaling (Alnabhani et al. 2015).

In the host, bacteria are exposed to various innate defense mechanisms, but many pathogenic germs, like *Shigella*, enteropathogenic *Escherichia coli*, *Listeria*, *Staphylococcus* or *Streptococcus*, are able to escape or interfere with these protection systems (Phalipon and Sansonetti. 2007; Tattoli et al. 2013; Long et al. 2014; Okumura and Nizet. 2014). We have previously found that *P. fluorescens* can induce and divert the human β -defensin-2 secretion in intestinal epithelial cells, leading to an increase in virulence (Madi et al. 2013). However, the enteric system, which is sometimes designated as the second brain, releases also a multitude of neurotransmitters and neurohormones, and the impact of these enteroendocrine molecules on *P. fluorescens* remains largely unknown.

In the present work, we investigated the effect of serotonin (5HT), substance P (SP) and epinephrine (Epi) on the growth, motility and virulence of the clinical strain *P. fluorescens* MFN1032.

Materials and methods

Bacterial strain

Pseudomonas fluorescens MFN1032 was collected from a pulmonary tract infection in a hospital of Haute-Normandie (France; Chapalain et al. 2008) and routinely cultivated at 28 °C, in ordinary nutrient broth (ONB; Merck, Darmstadt, Germany).

Bacterial growth with 5HT, SP and Epi

Overnight cultures of *P. fluorescens* MFN1032 were diluted in ONB to a final optical density (OD) of 0.08 at 600 nm. 5HT, SP or Epi were added to a final concentration of 10⁻³ or 10⁻⁶ M. Bacteria were then grown for 24 h with

a Bioscreen spectrophotometer using the following parameters: shaking, reading every 30 min, temperature of 28 °C. Each culture was grown in triplicate, and the experiment was repeated three times.

Swarming and swimming motility assays

To test on motility, a sterile needle was used to lightly touch bacterial cultures and spotted gently in the middle of a swarm plate (Luria–Bertani Broth, 0.6 % bacteriological agar) or a swim plate (Luria–Bertani Broth, 0.3 % bacteriological agar). The plates were incubated at 28 °C, and the migration of bacteria was measured after 24 h from the point of inoculation (observed as a turbid zone in centimeters).

Production of pyoverdinin

Pyoverdinin production of *P. fluorescens* MFN1032 was determined after 48 h of growth in Bacto Casamino Acids medium (CAA). The results were expressed as the OD_{400 nm}/OD_{600 nm} ratio of the supernatant after removal of the bacteria by centrifugation (5 min, 10,000g) as previously described (Dagorn et al. 2013).

Caco-2/TC7 cells culture

The human colon adenocarcinoma cell line Caco-2/TC7 was used between passages 40–60. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 15 % heat-inactivated fetal calf serum (FCS), 2 mM of L-glutamine, 100 U/ml each of penicillin and streptomycin and 1 % of nonessential amino acids. The cells were seeded and cultivated at 37 °C in 5 % CO₂–95 % air atmosphere, in 24-well tissue culture plates until confluence for cytotoxicity assay and quantification of IL-8; and on inserts (6.4 mm diameter, 3 μ m pore size, Falcon) until full differentiation (21 days) for transepithelial resistance measurements (TER) and actin staining.

Bacterial treatment with 5HT, SP or Epi and infection of Caco-2/TC7 cells

5HT, SP or Epi (10⁻⁶ M) were added to the cultures of *P. fluorescens* MFN1032 at the beginning of the exponential growth phase. After 4 h of exposition to these neuroendocrine factors, bacteria were collected by centrifugation for 5 min at 8000g, rinsed to remove any trace of the molecules and resuspended in DMEM without antibiotics and serum. Confluent or differentiated Caco-2/TC7 cells grown in 24-well plates or insert were then infected with control or treated bacteria at a MOI (multiplicity of infection) of 100:1 and incubated overnight at 37 °C in 5 % CO₂.

Cytotoxicity assay and quantification of IL-8

Supernatants from Caco-2/TC7 undifferentiated monolayers were collected after 18 h of infection. The concentration of lactate dehydrogenase (LDH), a cytoplasmic enzyme released upon cell death, was determined using the Cytotox 96 enzymatic assay (Promega, France). Total LDH release (100 % dead cells) was obtained by exposing Caco-2/TC7 cells to Triton X100 (0.9 %) as recommended by the manufacturer's instructions. IL-8 cytokine secretion was measured using ELISA Quantikine kit (R&D Systems, France).

Transepithelial resistance measurements

The transepithelial resistance (TER) of differentiated Caco-2/TC7 monolayers was measured after 18 h of bacterial infection, using the Millicell Electrical Resistance System (Millipore Corp, Bedford, MA). TER values are expressed as percentages of the initial level of the TER (baseline) measured for each individual cell monolayer in the inserts at the onset of the experiment.

Actin staining

At the end of infection, differentiated Caco-2/TC7 monolayers were washed with PBS, fixed for 10 min with 3.7 % paraformaldehyde and permeabilized for 5 min with 0.2 % Triton X100 at room temperature. The cells were then incubated with 1 % bovine serum albumin in PBS for 10 min, and the apical F-actin cytoskeleton was stained with Alexa-488 phalloidin (1 U/insert) for 45 min at room temperature. Following three washes in PBS, cell monolayers were examined using a confocal laser scanning microscope (Zeiss, LSM710) equipped with an argon laser for 488 nm excitation of Alexa-488. An oil-immersion objective lens (X63) was used, and imaging parameters standardized to allow direct comparison between images.

Statistical analysis

Data are expressed as mean \pm standard error (SE) calculated over a minimum of three separate experiments. Statistical significance was determined using Student's *t* test.

Results

Bacterial growth with 5HT, SP and Epi

The growth of *P. fluorescens* MFN1032 with 10^{-6} or 10^{-3} M of 5HT (Fig. 1a), SP (Fig. 1b) or Epi (Fig. 1c) was monitored for 24 h. The results show that the three molecules tested had only few effects on the growth of *P.*

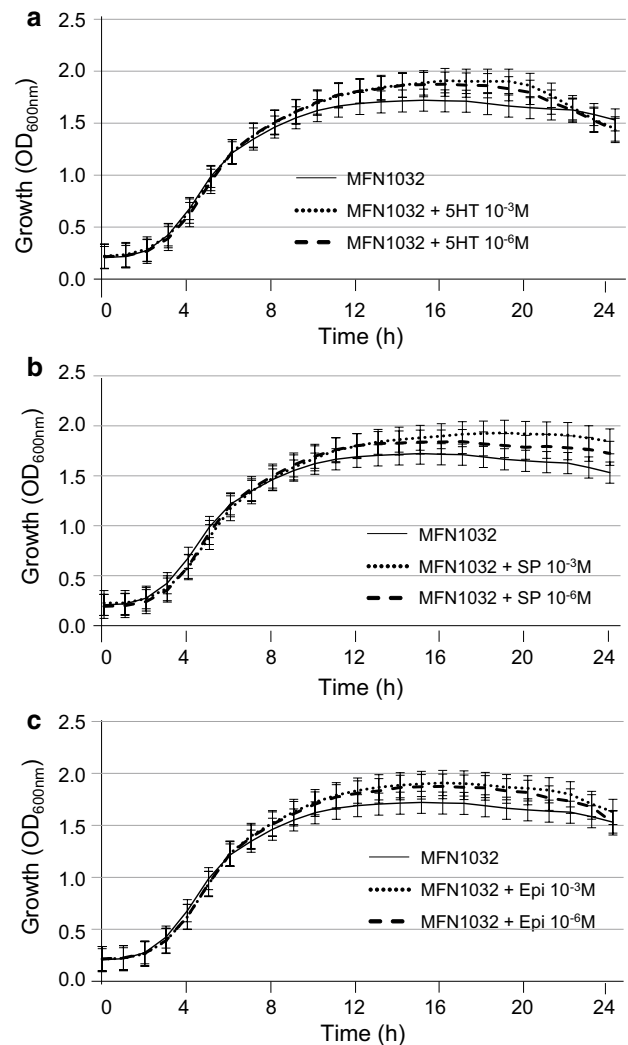


Fig. 1 Effect of 5HT, SP and Epi (10^{-6} and 10^{-3} M) on the growth of *P. fluorescens* MFN1032

fluorescens MFN1032. Lag phase and growth rate were not affected. This result indicates that if virulence is enhanced in the next experiments of our study, it could not be directly attributed to bacterial overgrowth.

Swimming and swarming motility tests

Swimming and swarming motilities were studied using 0.3 and 0.6 % agar in petri dishes. The migration distances of *P. fluorescens* MFN1032 pretreated or not with 5HT, SP or Epi were measured after 24 h incubation (Fig. 2). It appeared that only 5HT modulated significantly the swimming motility of *P. fluorescens* MFN1032. Indeed, untreated bacteria swam 5.7 ± 0.3 cm, whereas they reached 7.2 ± 0.3 cm after 5HT treatment. Conversely, the three molecules tested increased the swarming motility of *P. fluorescens* MFN1032, reaching 5.8 ± 0.3 , 6.3 ± 0.4

Fig. 2 Effect of 5HT, SP and Epi (10^{-6} M) on swimming (a) and swarming (b) motilities of *P. fluorescens* MFN1032. Y axis indicates the distance traveled (cm) by the bacteria. NS not significant, ** $P < 0.01$, *** $P < 0.001$, versus untreated bacteria

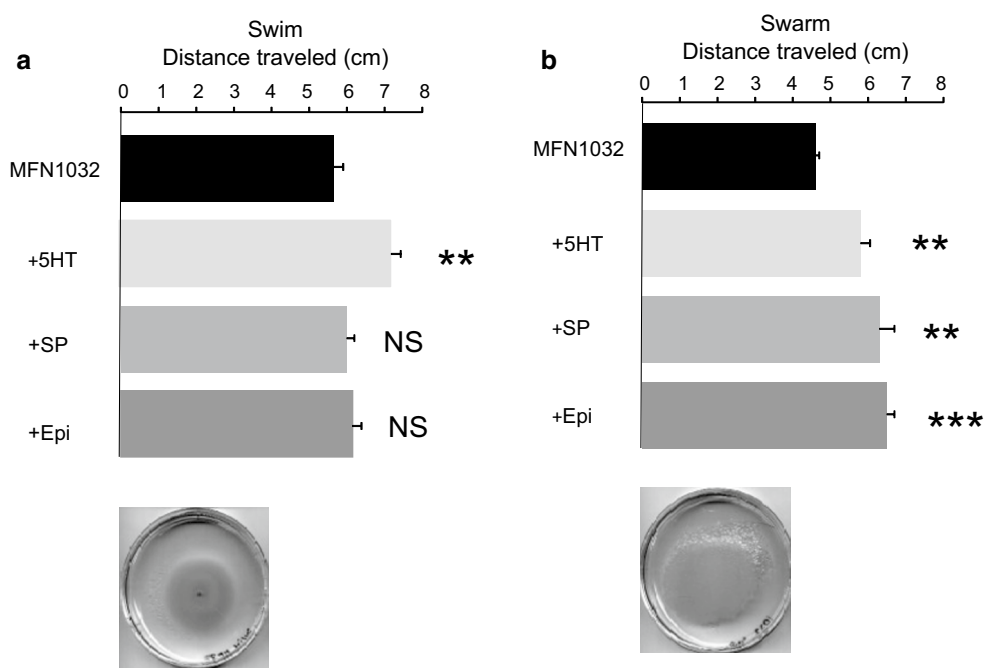
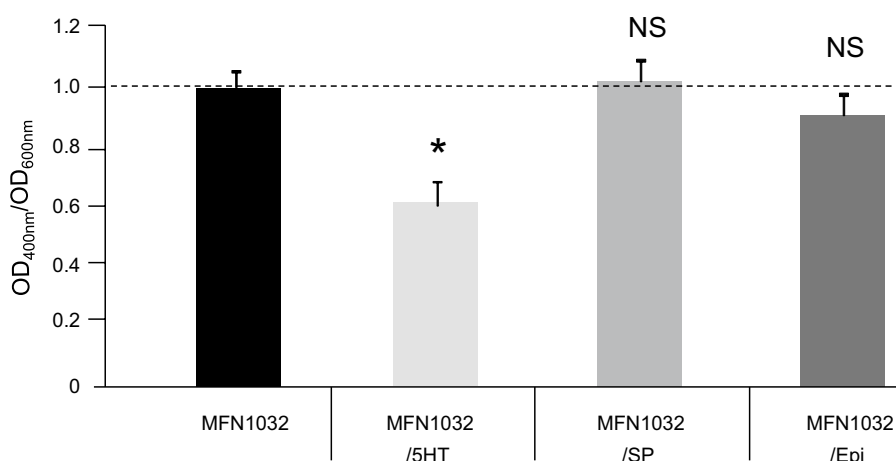


Fig. 3 Effect of 5HT, SP and Epi (10^{-6} M) on pyoverdinin production by *P. fluorescens* MFN1032. Pyoverdinin production is expressed as the ratio of pyoverdinin adsorption (OD_{400nm}) to the bacterial density (OD_{600nm}). NS not significant, * $P < 0.05$, versus untreated bacteria



and 6.5 ± 0.2 cm, in the presence of 5HT, SP and Epi, respectively, compared to 4.6 ± 0.1 cm for the untreated bacteria.

Production of pyoverdinin

Pyoverdinin is a siderophore and virulence factor essential for the colonization of bacteria belonging to *Pseudomonas* genus (Meyer et al. 1996). Here, we investigated the effect of 5HT, SP and Epi on the production of pyoverdinin by *P. fluorescens* MFN1032 after 48 h of bacterial culture (Fig. 3). The results show that 5HT induced a decrease in pyoverdinin production, whereas SP and Epi had no significant effect.

Cytotoxicity assay and IL-8 secretion

The cytotoxicity and pro-inflammatory potential of *P. fluorescens* MFN1032 treated with 5HT, SP or Epi were compared to untreated bacteria. We found that the cytotoxicity of *P. fluorescens* MFN1032 was not modified by any of the molecules tested (Fig. 4a), but when the bacteria were treated with SP before the infection of Caco-2/TC7 cells, the production of IL-8 was significantly increased (Fig. 4b).

Transepithelial electrical resistance measurements

The effect of *P. fluorescens* MFN1032 on differentiated Caco-2/TC7 cells was evaluated after 18 h of infection

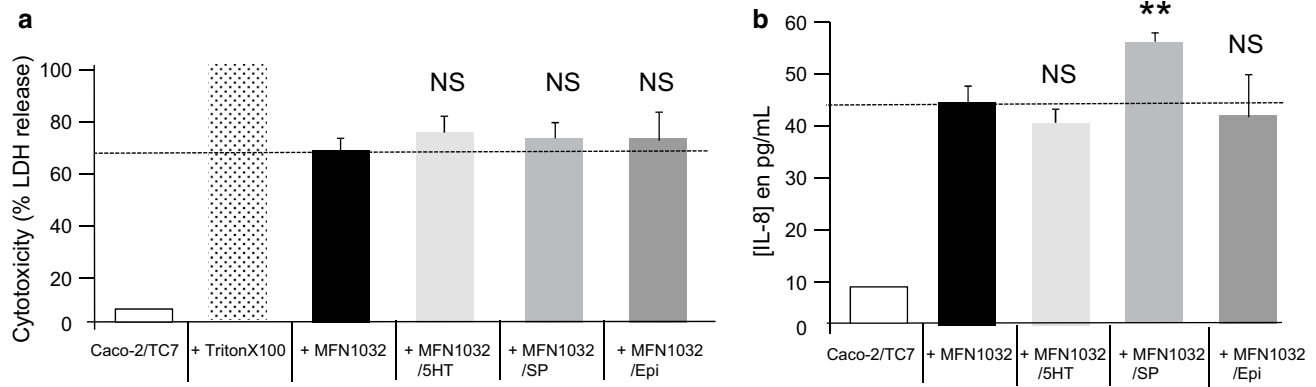
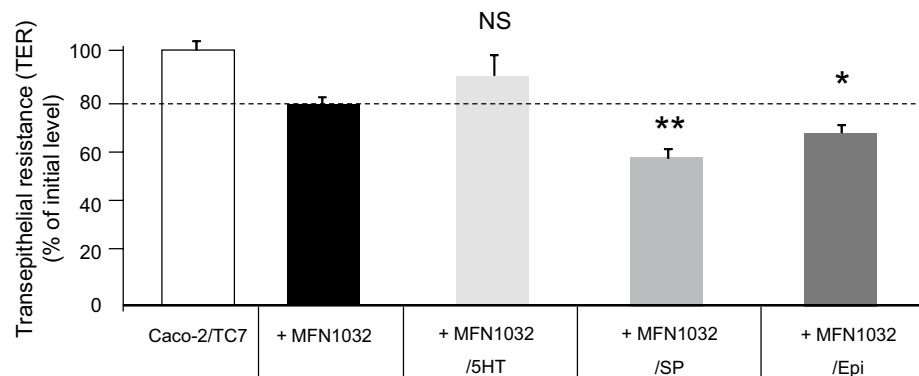


Fig. 4 Effect of 5HT, SP and Epi (10^{-6} M) on *P. fluorescens* MFN1032 cytotoxic activity toward Caco-2/TC7 cells (**a**) and pro-inflammatory potential: activation of interleukin-8 (IL-8) secretion

by Caco-2/TC7 cells (**b**). Cytotoxicity was evaluated by measurement of lactate dehydrogenase (LDH) released from damaged cells. NS not significant, ** $P < 0.01$, versus cells infected by untreated bacteria

Fig. 5 Effect of *P. fluorescens* MFN1032 treated or not by 5HT, SP and Epi (10^{-6} M) on the transepithelial resistance (TER) of differentiated Caco-2/TC7 cell monolayers after 18 h of infection. The data are expressed as percentage of the initial TER, measured across the monolayer at the onset of the experiment. NS not significant, * $P < 0.05$, ** $P < 0.01$, versus Caco-2/TC7 cell monolayers infected with untreated bacteria



(Fig. 5). The results show that the TER of the Caco-2/TC7 monolayers decreased more when the infection was done with the bacteria pretreated with SP or Epi than infection with the untreated bacteria. On the contrary, pretreatment of *P. fluorescens* MFN1032 with 5HT had no effect. This suggests that SP and Epi may enhance the damages of the monolayer provoked by the bacteria.

Actin staining

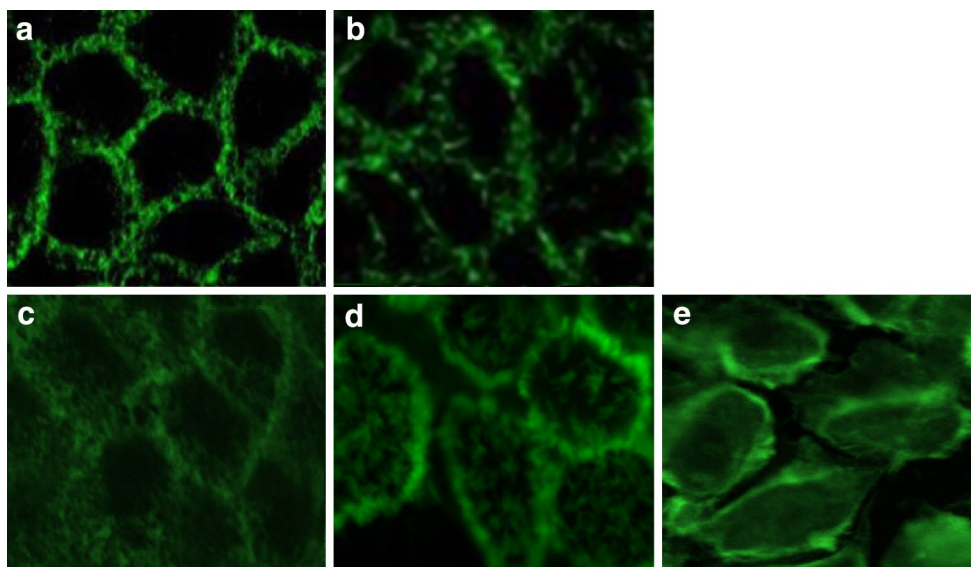
To visualize the results obtained by TER measurements, F-actin cytoskeleton was stained and observed by confocal microscopy. Differentiated Caco-2/TC7 cells showed a well-defined network of F-actin (Fig. 6a). When the monolayer was infected with *P. fluorescens* MFN1032, the F-actin appeared partly disorganized (Fig. 6b), and the same result was obtained when *P. fluorescens* MFN1032 was exposed to 5HT before infection of Caco-2/TC7 cells (Fig. 6c). Conversely, a pretreatment of the bacteria by SP (Fig. 6d) or Epi (Fig. 6e) led to the disruption of cell-to-cell contact of the epithelium and the redistribution of some F-actin filaments to the cytoplasm. These damages of the

Caco-2/TC7 monolayer are in agreement with the TER measurements.

Discussion

Although most widely studied for its role in the soil and the rhizosphere, *P. fluorescens* possesses a number of functional traits that provide it with the capability to grow and thrive in mammalian hosts. Multiple culture-based and culture-independent studies have identified it at low levels in the indigenous microbiota of various body sites. Although this germ is not generally considered as a bacterial pathogen in human, it has been associated with bacteremia and 54 % of Crohn's disease patients were found to develop serum antibodies to *P. fluorescens* (Sutton et al. 2000). This suggests that association between humans and *P. fluorescens* during health and disease may be complex. This bacterium was recently found to be able to induce and divert the human β -defensin-2 secretion in intestinal epithelial cells to enhance its virulence (Madi et al. 2013). However, the effect of other intestinal molecules produced by the

Fig. 6 Effect of *P. fluorescens* MFN1032 treated or not by 5HT, SP and Epi (10^{-6} M) on the F-actin cytoskeleton of Caco-2/TC7 cells. F-actin cytoskeleton was stained with Alexa Fluor® 488 phalloidin and examined using a confocal laser scanning microscope. Uninfected Caco-2/TC7 cells (a), Caco-2/TC7 cells infected with *P. fluorescens* MFN1032 (b), Caco-2/TC7 cells infected with *P. fluorescens* MFN1032 treated by 5HT (c), Caco-2/TC7 cells infected with *P. fluorescens* MFN1032 treated by SP (d), Caco-2/TC7 cells infected with *P. fluorescens* MFN1032 treated by Epi (e)



host on the virulence of *P. fluorescens* remains unknown. Our study shows for the first time that 5HT, SP and Epi can modulate the virulence behavior of the clinical strain *P. fluorescens* MFN1032, whereas its growth was feebly affected by these molecules even at high concentration level. Nevertheless, several eukaryotic molecules are known to be able to modulate positively or negatively the bacterial growth. High concentrations of SP ($>10^{-4}$ M) have previously been shown to display both direct and indirect antimicrobial activities on several bacteria including *E. coli* and *P. aeruginosa*, by acting as a weak cationic antimicrobial peptide (Kowalska et al. 2002; Hansen et al. 2006; Lesouhaitier et al. 2009). Conversely, 5HT added to an *E. coli* K12 culture stimulates cell proliferation and biomass accumulation (Anuchin et al. 2008), and the authors of this study showed a nonlinear concentration dependence curve probably due to a specific receptor-/sensor-dependent mechanism. 5HT was also found to enhance the growth and cellular aggregation of other bacterial species including *Streptococcus faecalis* or eukaryotic microorganisms, such as the yeast *Candida guilliermondii* (Strakhovskaia et al. 1993). Catecholamines, which include norepinephrine (NE), Epi and dopamine, are able to increase significantly the growth of *P. aeruginosa*, *K. pneumoniae* and *E. coli* (Belay and Sonnenfeld 2002), and this varies greatly among bacterial species (Belay et al. 2003). In our study, we found that even at concentrations much above those physiologically present in the host (10^{-3} M), the growth of *P. fluorescens* MFN1032 was not significantly affected by 5HT, SP and Epi, as previously observed for *P. fluorescens* with antimicrobial peptides (Madi et al. 2013), but also with γ -aminobutyric acid (GABA) (Dagorn et al. 2013) and natriuretic peptides (Veron et al. 2008).

We found that the modulation of virulence varied with the intestinal factor tested. SP and Epi were shown to increase the swarming motility of *P. fluorescens* MFN1032, while 5HT enhanced both the swimming and swarming capacities of the bacteria but led to a decrease in pyoverdinin production. In *E. coli* O157:H7 (EHEC), Epi and NE were found to increase motility and biofilm formation (Bansal et al. 2007). In fact, the AI-3 quorum-sensing system is involved in interkingdom signaling with the eukaryotic hormones Epi/NE. This AI-3/Epi/NE signaling system activates transcription of virulence genes in EHEC (Clarke et al. 2006) and is also found in other gram-negative bacteria like *Salmonella enterica* serovar Typhimurium for which this system is a key factor of in vitro and in vivo pathogenesis (Moreira et al. 2010). In *P. aeruginosa*, NE may replace siderophore by providing iron and this could repress the production of siderophores like pyoverdinin (Visca et al. 2002; Stintzi et al. 1999; Li et al. 2009).

None of the intestinal molecules tested significantly modulates the cytotoxicity of *P. fluorescens* MFN1032, but when the bacteria were pretreated with SP before infection of the Caco-2/TC7 cells, an increase in IL-8 secretion could be observed. Conversely, elevated necrotic activity has been found for *P. fluorescens* following treatment with natriuretic peptides (BNP, CNP) and GABA, and this was partially attributed to modifications in the LPS structure of the bacteria and surface properties (Veron et al. 2008; Dagorn et al. 2013). In addition, unlike our results, the cytotoxicity of *Bacillus cereus* has been found to be strongly stimulated after exposure to SP and increases in virulence in *Staphylococcus aureus* and *Staphylococcus epidermidis* were also observed (Mijouin et al. 2013).

A pretreatment of *P. fluorescens* MFN1032 with SP or Epi before infection of Caco-2/TC7 cells enhanced the permeability of the monolayer as shown by TER measurement and observation of the cells and F-actin by confocal microscopy. Indeed, Caco-2/TC7 monolayers were more damaged when infected with *P. fluorescens* MFN1032 pretreated by SP or Epi than after infection with the untreated bacteria. The same kind of effect was reported for Caco-2 cells infected with *Campylobacter jejuni* after treatment by NE (Cogan et al. 2007). Indeed, these authors found that when *C. jejuni* was grown in iron-limited media in the presence of NE, growth rate, motility and invasion of cultured epithelial cells were increased compared with cultures grown in the absence of the molecule. Bacteria exposed to NE during growth also caused greater subsequent disruption of cultured epithelial cell monolayers, inducing widespread breakdown of tight junctions.

In summary, our results demonstrate that the virulence behavior of *P. fluorescens* MFN1032 can be modulated by intestinal factors and this effect is molecule dependent. This suggests that intestinal molecules may be sensed by the gut microbiota and probably participate in health and disease. In the future, further studies should be necessary to investigate the mode of action of the enteroendocrine factors on *P. fluorescens* MFN1032 virulence and the modulation of the gut microbiota.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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-Article 8-



Structure-to-function relationships of bacterial translocator protein (TSPO): a focus on *Pseudomonas*

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The translocator protein (TSPO), which was previously designated as the peripheral-type benzodiazepine receptor, is a 3.5 billion year-old evolutionarily conserved protein expressed by most Eukarya, Archae and Bacteria, but its organization and functions differ remarkably. By taking advantage of the genomic data available on TSPO, we focused on bacterial TSPO and attempted to define functions of TSPO in *Pseudomonas* via *in silico* approaches. A *tspo* ortholog has been identified in several fluorescent *Pseudomonas*. This protein presents putative binding motifs for cholesterol and PK 11195, which is a specific drug ligand of mitochondrial TSPO. While it is a common surface distribution, the sense of insertion and membrane localization differ between α - and γ -proteobacteria. Experimental published data and STRING analysis of common TSPO partners in fluorescent *Pseudomonas* indicate a potential role of TSPO in the oxidative stress response, iron homeostasis and virulence expression. In these bacteria, TSPO could also take part in signal transduction and in the preservation of membrane integrity.

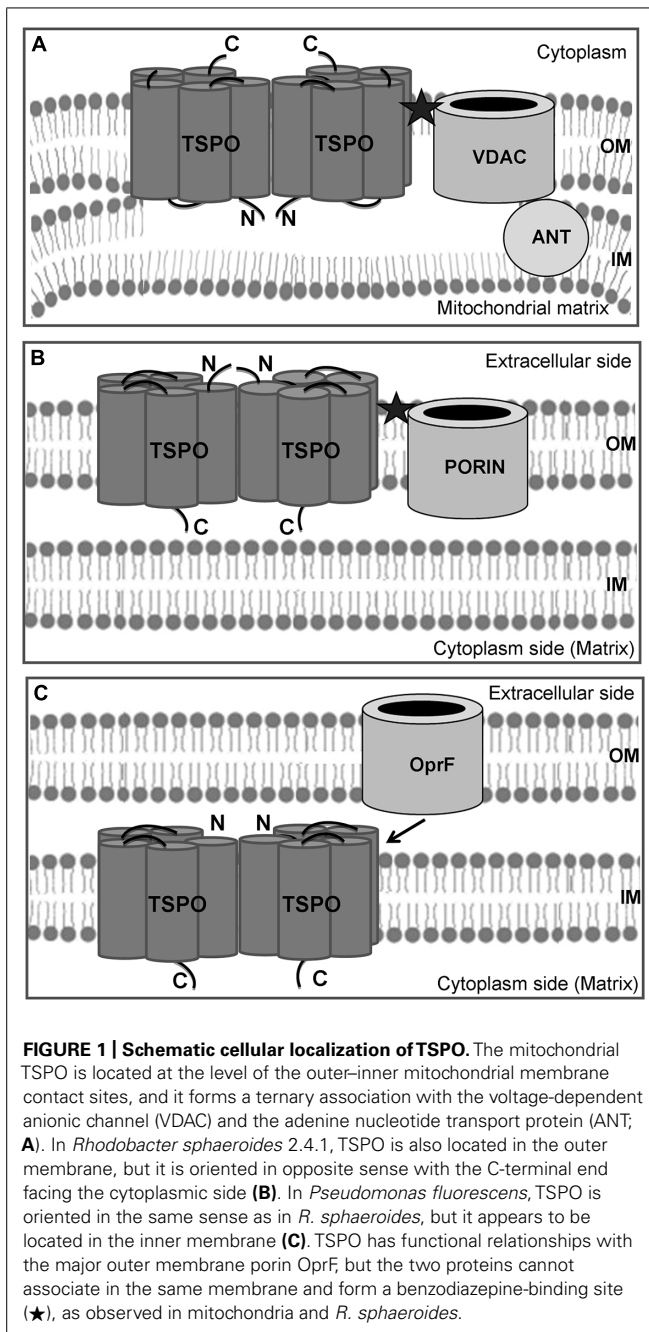
Keywords: translocator protein TSPO, bacteria, *Pseudomonas*, structure, function

INTRODUCTION

The translocator protein (TSPO), which was initially designated as the peripheral-type benzodiazepine receptor (PBR), was discovered as a diazepam-binding site in kidney (Braestrup and Squires, 1977), and found to be abundant in the outer mitochondrial membrane of steroid-synthesizing cells, including those in the central and peripheral nervous systems (Papadopoulos et al., 2006). Mitochondrial TSPO is associated to the voltage-dependent anionic channel (VDAC; also designated as mitochondrial porin) and to the adenine nucleotide transport protein adenine nucleotide translocase (ANT; McEnery, 1992; Papadopoulos et al., 2006, 2007) or the ATPase family AAA domain-containing 3A protein (Rone et al., 2012), forming a transmembrane hyperstructure (Figure 1A). TSPO has been involved in many physiological functions in mammals, including cell growth and proliferation, immunomodulation, mitochondrial respiration, apoptosis, and adaptation to oxidative stress (Wang et al., 1984; Ruff et al., 1985; Hirsch et al., 1989; Papadopoulos et al., 2006). One of its main functions is its implication in cholesterol import to the inner mitochondrial membrane, the rate-limiting step in steroid hormone biosynthesis (Papadopoulos et al., 1997a). TSPO has been shown to be a high affinity cholesterol-binding protein (Jamin et al., 2005), and to function together with other proteins in a complementary manner and within a large protein complex, to mediate the import of cholesterol into mitochondria (Miller and Bose, 2011; Issop et al., 2013; Poderoso et al., 2013). The function of TSPO as translocator or as acceptor of molecules (including cholesterol) associated with larger membrane translocator complexes is yet still unclear. TSPO drug ligands were shown to significantly affect cell and tissue steroid production and regulate

circulating and tissue steroid levels (Krueger and Papadopoulos, 1990; Papadopoulos et al., 1990, 1997b, 2006; Lacapère and Papadopoulos, 2003; Levine et al., 2007; Veenman et al., 2007; Rupprecht et al., 2009, 2010; Chung et al., 2013), indicating that TSPO is required for steroidogenesis. However, recent studies using a Leydig- and Sertoli-cell targeted knock out of the *tspo* gene (Morohaku et al., 2014) and a *tspo* null mice (Tu et al., 2014) indicated that the presence of TSPO may not be essential for steroid hormone biosynthesis. Although this raised controversy and several questions regarding mitochondrial TSPO function in steroidogenesis (Papadopoulos, 2014), and essential cell life processes, this does not detract from the fact that TSPO is abundant in steroidogenic cell mitochondria and that drug ligands act specifically on this protein to increase cholesterol import into mitochondria and steroidogenesis (Papadopoulos et al., 1997b, 2006; Lacapère and Papadopoulos, 2003; Levine et al., 2007; Veenman et al., 2007; Rupprecht et al., 2009). Recent structural studies confirmed the proposed structure and function of TSPO drug ligands (Jaremko et al., 2014). TSPO upregulation has been connected to several diseases, including cancer (Batarseh and Papadopoulos, 2010), neuronal damage, neurodegeneration, and inflammation, making the protein an important marker for glial cell activation and neuroinflammation (Harberts et al., 2013; Dickens et al., 2014). Recently, TSPO has attracted attention as a possible molecular target for tumor imaging and chemotherapy (Austin et al., 2013), and initial clinical trials have indicated that TSPO ligands might be valuable in the treatment of neurological and psychiatric disorders (Rupprecht et al., 2010).

Considering these important functions and perspectives in mammals, TSPO has been the focus of multiple studies and



reviews (Papadopoulos et al., 2006; Rupprecht et al., 2010; Fan et al., 2012). However, it remains a mysterious protein, since functional links between its putative translocation activity and its implication in multiple physiological functions are still lacking (Fan et al., 2012). Since TSPO was conserved throughout evolution in among the great diversity of Eukarya, Archaea, and Bacteria (Chapalain et al., 2009; Fan et al., 2012), and since bacterial and mammalian TSPOs are functionally interchangeable (Yeliseev et al., 1997), it is tempting to assume that the more ancestral cell forms – the bacteria – could provide further insights into the TSPO structure-to-activity relationships. The present study aims to describe bacterial TSPO in terms of its structure and function

on the basis of published data and *in silico* predictive approaches, with a special focus on TSPO belonging to the highly adaptable *Pseudomonas* genus.

BACTERIAL TSPO STRUCTURE

The first bacterial protein homolog of PBR was described in the non-sulfurous photosynthetic purple bacterium *Rhodobacter sphaeroides* by Yeliseev and Kaplan (1995). In that paper, the nomenclature tryptophan-rich sensory protein (TSPO) was proposed. The 17 kDa bacterial protein is composed of 158 amino acids and shows only 33.5 and 21% of similarity and identity respectively with human TSPO (Yeliseev et al., 1997). Thanks to molecular modeling, *R. sphaeroides* TSPO was proposed to fold into the outer membrane (OM) as five hydrophobic α helix regions, similarly to eukaryotic TSPO (Yeliseev and Kaplan, 2000), suggesting that the protein was conserved at the topological rather than at the amino acid sequence level. The postulated functional form of *R. sphaeroides* TSPO is a homodimer (Yeliseev and Kaplan, 2000). The three-dimensional structure of mammalian TSPO in complex with the PK 11195 drug ligand was recently reported and confirmed the previously reported TSPO topography, drug and cholesterol-binding sites as well as provided a model for ligand-induced cholesterol transport (Jaremko et al., 2014). However, as shown in **Figures 1A,B**, the *R. sphaeroides* and mitochondrial TSPOs are proposed to be inserted in opposite directions, i.e., the N-terminus is exposed to the extracellular side of the bacterial protein (Yeliseev and Kaplan, 2000), while it is located at the interface of the inner and outer mitochondrial membranes. One hypothesis that may account for this particularity is that while in bacteria, the protein is produced in the cytoplasm and then addressed into the OM from the inside, in eukaryotes the *tspo* gene has been translocated to the nuclear DNA (Gray et al., 2001) probably a billion years ago; therefore, TSPO is synthesized in the cell's cytoplasm and inserted in the mitochondrial membrane from the outside via translocase Tom70 (Otera et al., 2007) and metaxin 1 (Rone et al., 2009). The localization of TSPO in the OM of *R. sphaeroides* is further consistent with the observation that benzodiazepines such as flunitrazepam can crosslink TSPO to its major OM porin, as occurs in mammals (Yeliseev and Kaplan, 1995). This association may indeed improve the recognition of benzodiazepines in mammals, since the binding site for these artificial ligands could be located in the cleft between the two proteins (**Figure 1**, black star; McEnery, 1992; Lacapère et al., 2001; Veenman et al., 2007), albeit TSPO alone could be involved in the mitochondrial protoporphyrin IX (PPIX) import (Wendler et al., 2003).

FUNCTIONS

As in Eukarya, the postulated roles of TSPO in bacteria are numerous, but we can begin to identify their common general functions. The first periplasmic loop of *R. sphaeroides* TSPO contains a high percentage (22%) of tryptophan residues, reflecting possibly a WWD heme-binding domain that is typical of proteins involved in heme membrane transport (Goldman et al., 1998). Mitochondrial TSPO was previously shown to bind PPIX (Kinnally et al., 1993; Taketani et al., 1995), suggesting that TSPO may have a role

in mitochondrial processing of PPIX via the heme synthesis pathway (Furre et al., 2005; Veenman et al., 2007). In *R. sphaeroides*, TSPO appears to be involved in controlling the efflux of tetrapyrrole intermediates of the heme/bacteriochlorophyll biosynthetic pathway, and it could act as a negative regulator of photosynthetic gene expression and pigment synthesis in response to variations of oxygen and/or light availability (Yeliseev and Kaplan, 1995). The mechanism by which TSPO in *Rhodobacter* controls the transport of porphyrins remains to be determined. Interestingly, in the plant *Arabidopsis thaliana* (At), AtTSPO has been proposed to participate in the interactions between the plastidal and the mitochondrial tetrapyrrole biosynthetic pathway (Lindemann et al., 2004). This complex function of TSPO in the response to environmental conditions was confirmed in *R. capsulatus*, where TSPO is highly expressed in anaerobic conditions and in the absence of light (Bauer, 2004). TSPO indeed down-regulates transcription of bacterio-chlorophyll and carotenoid biosynthesis genes, as well as the *puc* operon, which encodes the structural proteins of the light-harvesting-II peripheral antenna complex (Yeliseev et al., 1997). The role of TSPO appears to be similar in cyanobacteria since it was shown to control photosynthetic genes expression in *Synechococcus* (González et al., 2011). Similarly, TSPO could be involved in the rapid shut down of the photosynthesis gene cluster in response to oxygen variations in the phototrophic bacterium, *Dinoroseobacter shibae* (Tomasch et al., 2011), and in the regulation of gene expression by light in the marine flavobacterium *Dokdonia* (González et al., 2011). More generally, TSPO has been proposed by Yeliseev et al. (1997) as an “oxygen sensor.” This protein could control porphyrin efflux by modulating the antirepressor/repressor AppA/PpsR system implicated in the regulation of photosynthetic gene expression in response to changes in oxygen partial pressure (Oh and Kaplan, 2001; Zeng and Kaplan, 2001). Noticeably, this activity is not specific to bacterial TSPO, since a mitochondrial *tspo* gene provided *in trans* in *R. sphaeroides* can replace the bacterial TSPO oxygen sensor function (Yeliseev et al., 1997). Interestingly, in the symbiotic *Sinorhizobium meliloti*, TSPO and FixL, an oxygen sensor, are involved in regulating the expression of the *ndi* locus that is specifically induced in nutrient (oxygen, carbon, nitrogen) deprivation conditions. TSPO appears to be epistatic to FixL, since no expression is observed in the *tspo* mutant, even though FixL is still present (Davey and de Bruijn, 2000). Further investigations are necessary to determine if these related TSPO homologs function in a similar manner and respond to the same signals. Nonetheless, these data suggest that TSPO is involved in regulating gene expression and is likely to provide a new and important way to think about signal transduction in prokaryotes.

TSPO IN *Pseudomonas* GENOMIC ORGANIZATION, SEQUENCES, AND TOPOLOGY CONSERVATION

The structure–activity relationships of TSPO have been particularly investigated in *Pseudomonas fluorescens* MF37 (Chapalain et al., 2009). Here we have identified a *tspo* ortholog gene in 7 of the 48 fully sequenced genomes of *Pseudomonas*¹ (Winsor et al.,

2011), among which are three *Pseudomonas syringae* (*pv. phaseolicola* 1448A, *pv. syringae* B728a, and *pv. tomato* DC3000), three *Pseudomonas fluorescens* (strains SBW25, Pf10-1, A506), and one *Pseudomonas poae* (strain RE*1-1-14). *In silico* analysis will thus be focused on these strains. The genomic environment of *tspo* is not conserved among these seven strains, as previously described (Chapalain et al., 2009), although some degree of conservation exists in each species. For example, in two of the three studied *Pseudomonas fluorescens* strains, SBW25 and A506, *tspo* forms an operonic structure with an esterase-encoding gene that is involved in lipid metabolism¹ (Winsor et al., 2011). Many transposase-encoding genes were found in the vicinity of *tspo* in the three *Pseudomonas syringae* strains. Transposases are usually included in autonomous mobile genetic elements, such as transposons or insertion sequences, and they are required for excising and inserting the mobile element, suggesting that in these three strains, *tspo* acquisition may be the result of an ancient horizontal transfer. Moreover, TSPO could be functionally related to PSPPH_2782 (a transposase of *Pseudomonas syringae pv. phaseolicola* 1448A²), but the guanine–cytosine (GC) ratio of *Pseudomonas syringae tspo* is the same as in the rest of its genomic environment, suggesting that if *tspo* was acquired by insertion, the event was ancient.

Base-pairing comparisons using the MultAlin algorithm³ showed that TSPO was highly conserved among these seven strains at both the nucleotide and amino acid sequence levels, with similarities ranging from 71.6–91.1 and 76–95.9%, respectively. Protein secondary motifs were also conserved in *Pseudomonas* since a TopPred analysis⁴ revealed that TSPO folds into five putative transmembrane α -helices (Figure 2: T1–T5), similarly to *R. sphaeroides* and mitochondrial TSPO (Chapalain et al., 2009).

However, *Pseudomonas* TSPO was predicted to fold into the inner membrane (Figure 1C; Chapalain et al., 2009), whereas *R. sphaeroides* TSPO was predicted to insert in the OM as mitochondrial TSPO (Figure 1B, Yeliseev and Kaplan, 2000). This is consistent with the observation that in *Escherichia coli*, a recombinant eukaryotic protein was essentially retrieved from inner membrane extracts (Yeliseev et al., 1997), suggesting the different localization of TSPO in α - and γ -proteobacteria (Chapalain et al., 2009). Furthermore, the orientation of this insertion was in the opposite direction with respect to *Pseudomonas* and *R. sphaeroides* TSPO: in the case of *Pseudomonas* TSPO, the N-terminus was inserted into the inner membrane; conversely, it is exposed at the bacterial surface in *R. sphaeroides* (Figures 1B,C). Interestingly, in mammals, TSPO was also found in the cytoplasmic membrane (Oke et al., 1992; Woods and Williams, 1996; Woods et al., 1996), suggesting that TSPO could exert functions at different levels, including on the cell surface or outside the cell.

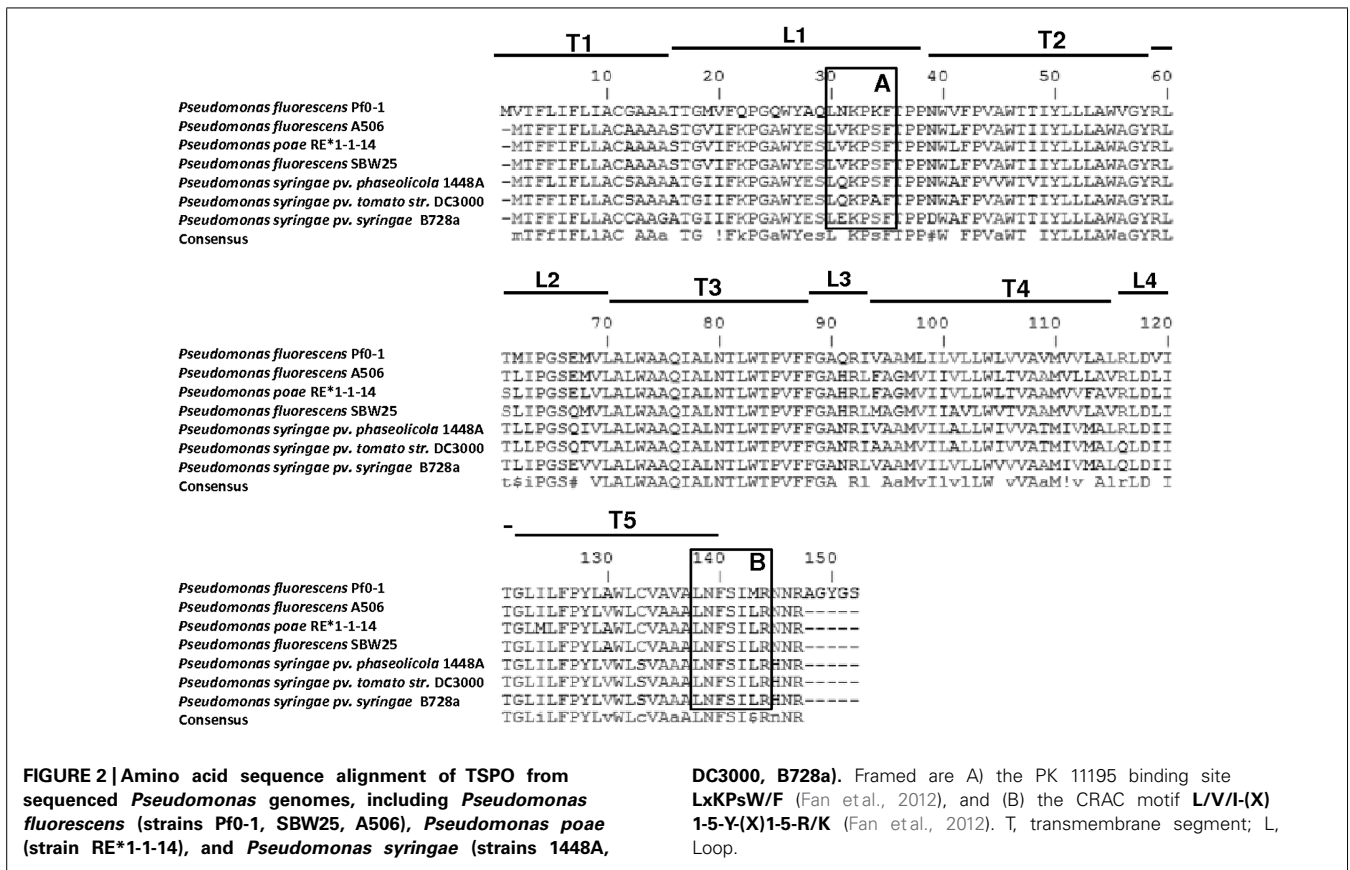
In addition, the potential PK 11195 binding motif LxKPsW/E, previously determined by Fan et al. (2012), was identified in the first loop (Figure 2). This motif was fully conserved among the seven TSPO sequences of fluorescent *Pseudomonas*, suggesting it

¹<http://www.ncbi.nlm.nih.gov/gene/3559346>

²<http://multalin.toulouse.inra.fr/multalin/multalin.html>

³<http://www.pasteur.fr/>

⁴<http://www.pseudomonas.com>



may play a function in *Pseudomonas* TSPO. This binding motif was mapped onto the recently reported 3D structure of mouse TSPO just outside of the PK 11195 binding pocket and it consists of 65 contacts between the ligand and TSPO involving the five transmembrane domains (Jaremko et al., 2014). PK 11195 is an isoquinoline carboxamide drug ligand of the mitochondrial TSPO, whose binding site is exclusively located on TSPO (Le Fur et al., 1983). The use of PK 11195 in *Pseudomonas fluorescens* MF37 allowed researchers to demonstrate that there was a functional association between TSPO and the major OM porin OprF since the biological activity of PK 11195 was totally abolished in an OprF deletion mutant (Chapalain et al., 2009). Moreover, the affinity of PK 11195 for *Pseudomonas fluorescens* MF37 TSPO was the same as for mitochondrial TSPO, indicating the preservation of common properties between the two proteins (Chapalain et al., 2009). However, in normal and low-oxygen conditions, PK 11195 was without effect on the growth kinetics of *Pseudomonas fluorescens* MF37 (Chapalain et al., 2009), suggesting that it should not act as an oxygen sensor as in *R. sphaeroides* (Yeliseev et al., 1997). The localization of TSPO in the OM of *R. sphaeroides* is consistent with the observation that benzodiazepines such as flunitrazepam can crosslink TSPO to its major OM porin, as in mammals (Yeliseev and Kaplan, 1995). While in mitochondria TSPO and VDAC are in the same (outer) membrane where they can associate to form a diazepam-binding site (Figures 1A,B), in *Pseudomonas fluorescens*, TSPO and porins (such as OprF) should be inserted in the inner and OMs, respectively (Figure 1C). This can explain the

absence of sensitivity of *Pseudomonas fluorescens* to diazepam (our unpublished data) since, if TSPO also interacts with porin(s), the structure of the complex should be different. The conservation of the PK 11195 binding motif in the bacterial TSPO sequences suggests that molecules mimicking the structure of PK 11195 might interact with TSPO. The association of bacterial TSPO with OprF in case of *Pseudomonas fluorescens* MF37, or the major OM protein in case of *R. sphaeroides* further suggest that TSPO might be the binding site for such molecules and that it is associated with larger membrane channels, relatively similarly to mitochondrial TSPO associated to VDAC.

Taken together, these data show that *Pseudomonas* TSPO shares common structural properties with *R. sphaeroides* and mitochondrial TSPOs. It should be noted that the most intriguing observation included the topological differences found among several of the proteins between eukaryotic and prokaryotic TSPOs; however, the most striking difference was seen between *R. sphaeroides* and *Pseudomonas* TSPOs, suggesting that they hold common yet specific functions.

PUTATIVE FUNCTIONS

The functional role of TSPO in these bacteria appears to be at least as essential as in *R. sphaeroides*, since while in this species a knock-down of TSPO is possible (Yeliseev and Kaplan, 1995), any major change of TSPO expression (positively or negatively) in all investigated strains of *Pseudomonas fluorescens* is lethal (Chapalain et al., 2009).

TSPO and cholesterol/steroidogenesis

As described in the introduction section, one of the functions of mitochondrial TSPO is its involvement in cholesterol import into mitochondria, which is a prerequisite for steroidogenesis – a function which was recently questioned due to the absence of observed effects on steroidogenesis in a *tspo*-null mice mutant– (Morohaku et al., 2014; Tu et al., 2014). Although these findings argue that cholesterol import into mitochondria may occur in the absence of TSPO, they do not indicate that in normal cells this process is not mediated by TSPO, a protein abundant in the outer mitochondrial membrane. Noticeably, the potential cholesterol recognition amino-acid consensus (CRAC) L/V/I-(X)_{1–5}-Y-(X)_{1–5}-R/K, previously determined in mammals by Li and Papadopoulos (1998), and shown to be common among many proteins (Fantini and Barrantes, 2013), and its evolution reviewed by Fan et al. (2012), was identified in the C-terminal part of TSPO (Figure 2). The CRAC motif lacked the internal characteristic Y of eukaryotes (Figure 2). This feature, associated with the absence of cholesterol in bacterial membranes, prompted Fan et al. (2012) to suggest that this motif is rarely found in bacterial TSPO sequences. Notably, this sequence was highly conserved among the seven *Pseudomonas* TSPOs, suggesting that this C-terminal part has a special function in the TSPO role in these bacteria. Cholesterol has not yet been found to be produced in bacteria. However some steroids, among which cycloartenol, the first oxidosqualene cyclization product in plants (Bode et al., 2003), or lathosterol, a molecule closely structurally-related to cholesterol, have been shown to be produced by several *Myxobacteria* species as well as by *Methylococcus capsulatus* (Bird et al., 1971; Tippelt et al., 1998; Bode et al., 2003; Gawas et al., 2011). Moreover, a phylogenetic study of the sterol biosynthesis pathways reported that some other bacterial genomes may possess certain genes which products could be involved in sterol biosynthesis pathways. In particular, this study reported that *Myxobacterium Plesiocystis pacifica* harbors the largest reported set of eukaryotic sterol-synthesizing enzyme homologs (Desmond and Gribaldo, 2009), suggesting that this *Myxobacterium* could be at the origin of these eukaryotic enzymes (López-García and Moreira, 1999). It is also likely that *Plesiocystis pacifica* has acquired its pathway for sterol synthesis via horizontal gene transfer from eukaryotes (Desmond and Gribaldo, 2009).

In the *Myxobacteria Nannocystis exedens*, almost all known intermediates and side-products of the cholesterol biosynthesis in eukaryotes can be found under different culture conditions (Bode et al., 2003). Although the authors clearly demonstrate that *Myxobacteria* can produce as much steroids as eukaryotic organisms, the function of these compounds remains a mystery. Due to their hydrophobicity, these molecules are most probably located in membranes, where they might have a function in controlling fluidity, in a manner similar to that described for cholesterol in eukaryotes (Ourisson and Nakatani, 1994). An additional function of the myxobacterial steroids might be to act as signaling molecules, similar to steroid hormones (Porter et al., 1996). Interestingly, a TSPO homolog has been predicted in some *Myxobacteria* species⁵.

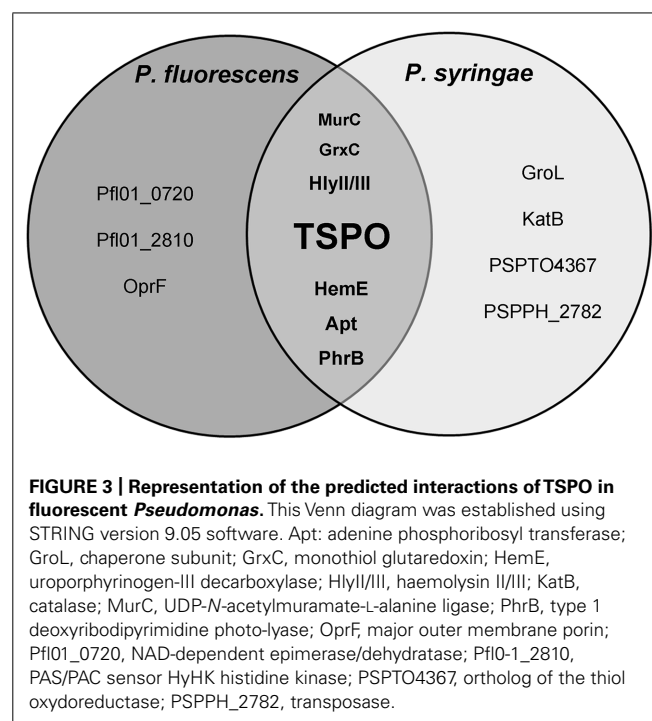
⁵<http://www.uniprot.org/>

To date, no *Pseudomonas* species has been shown to produce such compounds, and the genes encoding the required enzymes are actually not predicted in the *Pseudomonas* database¹. It may be conceivable that these bacteria could perceive or transport exogenous cholesterol or even structurally-related tetracyclic molecules like hopanoids for example (Fan et al., 2012). It is also possible that the functions of the *Pseudomonas* TSPO were evolutionary older, and that the possible involvement of mitochondrial TSPO in cholesterol transport and steroidogenesis occurred much later.

TSPO and iron homeostasis

To get further insights into the putative protein functions in *Pseudomonas*, TSPO's functional interactions were retrieved from STRING database (von Mering et al., 2007), in *Pseudomonas syringae* (strains B728a, 1448A and DC3000) and *Pseudomonas fluorescens* (strains SBW25, Pf10-1) since STRING data were not available for either *Pseudomonas fluorescens* A506 or *Pseudomonas poae* RE*1-1-14. STRING is a database of known and predicted protein interactions, including physical and functional associations, which are derived from four sources including genomic context, high-throughput experiments, coexpression and literature. Only interactions with high confidence levels (>0.7) were kept. Common TSPO partners in each of the five studied strains were then represented as a Venn diagram (Figure 3; Chen and Boutros, 2011). This STRING-based study should be understood as a first hint where to search.

HemE is found to interact with TSPO in each of the studied strains, as well as in *R. sphaeroides* and *Sinorhizobium meliloti* (STRING), suggesting a putative robust link between the two proteins. HemE is an uroporphyrinogen-III decarboxylase that produces coproporphyrinogen III, also called apoferritin. Ferritin



and ferritin-like molecules store Fe^{3+} as a mineral in their hollow cavities, effectively concentrating iron levels to orders of magnitude higher than those permitted by its low solubility (Yao et al., 2011). Iron is required by most organisms, but it is potentially toxic due to its low solubility, and to its tendency to potentiate the production of reactive oxygen species (ROS). The reactivity of iron is counteracted by bacteria using the same strategies employed by eukaryotes, namely by sequestering the metal into ferritin. These pivotal contributions made by ferritin-like molecules to iron homeostasis are manifested by their presence in all three domains of life with remarkable conservation of structure and function, despite very low sequence conservation (<20%; Grossman et al., 1992).

In addition to this major function in iron scavenging, apo-ferritin is also an intermediate of porphyrin and heme biosynthesis. Notably, in *R. sphaeroides*, TSPO has been shown to act as a negative regulator in the expression of *hemN* (Yeliseev and Kaplan, 1999), encoding a protein involved in heme trafficking. In mammals, coproporphyrinogen III is transported into the mitochondrial space where its conversion to PPIX takes place. PPIX is then converted into heme, which then sequesters iron (Fe^{2+}), thus forming heme (Kabe et al., 2006). The intra-mitochondrial accumulation of PPIX in response to iron starvation is toxic, and TSPO has been involved in mitochondrial detoxification, at least partly through PPIX export (Yeliseev and Kaplan, 1999; Mesenholler and Matthews, 2000). Remarkably, in the plant *A. thaliana*, in which heme biosynthesis is mainly localized in plastids (Mochizuki et al., 2010), TSPO has been proposed to participate in the interaction between the plastidal and the mitochondrial tetrapyrrole biosynthetic pathway, most likely by transporting the protoporphyrinogen from the plastids to the mitochondrial site of heme formation (Lindemann et al., 2004). Plant TSPO is also involved in scavenging unbound heme and porphyrins (Vanhee et al., 2011), and has recently been proposed to function as an autophagy receptor for toxic porphyrins leading to their specific degradation (Veljanovski and Batoko, 2014). Recently, bacterial TSPOs have been shown to catalyze rapid porphyrin degradation in a light- and oxygen-dependent manner – a reaction that is inhibited by the synthetic TSPO ligand, PK 11195 – and by mutations of conserved residues, which affect either porphyrin binding or catalytic activity (Ginter et al., 2013). Taken together these data suggest that TSPOs are ancient evolutionary conserved enzymes mediating porphyrin catabolism most likely with the consumption of ROS (Ginter et al., 2013).

TSPO and oxidative stress response

Iron is both an essential nutrient for the growth of microorganisms, as well as a dangerous metal due to its capacity to generate ROS via the Fenton reaction. For these reasons, bacteria must tightly control the uptake and storage of iron in a manner that restricts the build-up of ROS. Therefore, the control of iron homeostasis and responses to oxidative stress are intimately coordinated (Cornelis, 2010). The link between TSPO and the response to ROS is strengthened by the fact that several proteins that are involved in the oxidative stress response were predicted to interact with *Pseudomonas* TSPO. This was the case for example of

the monothiol glutaredoxin GrxC that was found in fluorescent *Pseudomonas* TSPO interactome (Figure 3), which is involved in the bacterial oxidative stress response and in iron homeostasis (Cabisco et al., 2000). In *E. coli*, GrxC is able to bind to glutathione, helping to maintain the redox status of the cell (Lillig et al., 2008). GrxC can then reduce the oxidized redox-response master regulator OxyR, thus creating a feedback loop that regulates OxyR activity (Zheng et al., 2001). In addition, GrxC is also a player in iron homeostasis, since it has been shown to bind Fe–S clusters, and it is also involved in some aspects of Fe–S cluster biogenesis and regulation (Iwema et al., 2009; Couturier et al., 2011; Boutigny et al., 2013). In the fungi, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, GrxC also plays essential roles in intracellular iron signaling, iron trafficking, and maturation of Fe–S cluster proteins (Mühlenhoff et al., 2010; Mapolelo et al., 2013). Common to each of the studied *Pseudomonas* TSPO functional networks is also PhrB (Figure 3), a conserved photolyase, the structure of which contains a [4Fe–4S] cluster as a cofactor bound to the catalytic domain (Zhang et al., 2013). Of note, PhrB was also predicted to interact with *R. sphaeroides* TSPO (STRING). Photolyases are enzymes involved in DNA repair and cell cycle regulation under stress conditions, which are commonly associated with the bacterial SOS response (Aravind et al., 2013). Interestingly, in mammals, mitochondria are the central cellular compartment for Fe–S cluster biogenesis, which is implicated in mitochondrial respiration and DNA repair (Stehling et al., 2014). Thanks to their chemical versatility, Fe–S centers act as catalysts or redox sensors (Stehling et al., 2014), and they are thought to rank among the most ancient and versatile inorganic cofactors found in all kingdoms of life (Beinert et al., 1997). The link between TSPO and ROS is further strengthened by the predicted interactions between *Pseudomonas syringae* TSPOs and both catalase KatB and orthologs of the thiol oxydoreductase PSPTO4367 (Figure 3), which are two key enzymes in almost all living organisms that are used to protect themselves against oxidative stress (Cabisco et al., 2000; Mishra and Imlay, 2012). Interestingly, KatB was also linked to TSPO in *Sinorhizobium meliloti* (STRING data not shown).

In mammals, the mitochondrial location of the TSPO is interesting as it is well known that mitochondria are a main source of cellular ROS (Lenaz, 1998). It has been shown that oxidative stress modulates TSPO structure and function (Delavoie et al., 2003). Increased ROS levels lead indeed to TSPO polymerization through di-tyrosine formation, which modulates the function of TSPO in cholesterol transport, since polymer formation induced by ROS increased both TSPO ligand binding and cholesterol-binding capacities (Delavoie et al., 2003). Vice versa, TSPO appears to be an essential participant in ROS generation at mitochondrial levels (Veenman et al., 2007; Zeno et al., 2009; Choi et al., 2011). In the liver, TSPO was found in co-localization with a ROS scavenger, the mitochondrial manganese-dependent superoxide dismutase (SOD; Fischer et al., 2001). In the moss plant *Physcomitrella patens* (Pp), *PpTspo1* knockout mutants were shown to be impaired in their mitochondrial PPIX uptake and produced elevated levels of intracellular ROS, implying a role of PpTSPO1 in redox homeostasis (Frank et al., 2007; Vanhee et al., 2011). TSPO-deficient plants were shown to be under permanent oxidative stress and

suffer from disturbed redox homeostasis (Lehtonen et al., 2012). Abiotic stresses, among which salt, drought or cold, transiently up-regulate heme biosynthesis that is required for the activity of ROS scavengers, and simultaneously induce the expression of TSPO (Frank et al., 2007). In *A. thaliana*, AtTSPO is expressed in dry seeds and can be induced in vegetative tissues by osmotic and salt stresses or by abscisic acid (ABA) treatments, suggesting that AtTSPO is specifically induced by water-related stress (Guillaumot et al., 2009a; Balsemão-Pires et al., 2011). Interestingly, the stress phytohormone ABA regulates plant water status through regulation of stomatal closure (Finkelstein et al., 2002; Nambara and Marion-Poll, 2005). The increase in active ABA levels in plant cells during water-related stress regulates the expression of ABA-responsive genes, among which *tspo* (Guillaumot et al., 2009a,b; Balsemão-Pires et al., 2011).

Taken together, these data suggest that *Pseudomonas* TSPO belongs to an evolutionarily conserved coordinated network that is involved in controlling iron and redox homeostasis.

Virulence

TSPO was predicted to interact with hemolysin II/III (Figure 3, HlyII/III). These proteins are exoenzymes that exhibit cytolytic activity against eukaryotic cells, which are involved in retrieving iron from eukaryotic heme (Guillemet et al., 2013). Such enzymes belong to the virulence factor arsenal of pathogenic bacteria, which enable bacterial survival among hosts in which the iron concentration is limited (Cornelis, 2010). The link between TSPO and virulence is also supported by a recent transcriptomic study in which *tspo* expression was increased in *Pseudomonas syringae* sp. *phaseolicola* NPS3121 cultured at a low (18°C) rather than at an optimal (28°C) growth temperature (Arvizu-Gomez et al., 2013). Interestingly, low temperature is the cue for pathogens to produce virulence factors, including toxins, cell wall-degrading enzymes, and effector proteins (Picot et al., 2004; Arvizu-Gomez et al., 2013), suggesting that TSPO may be part of a virulence-related network. It is interesting to note that in *Pseudomonas syringae* pv. *syringae* B728a and in *Pseudomonas syringae* pv. *tomato* DC3000, *tspo* is located in the vicinity near genes that encode homologs of virulence factors, SrfB and SrfC, of *Salmonella enterica* (Chapalain et al., 2009). These data are also in agreement with those showing that TSPO should be related to the expression of the cytotoxic activity of *Pseudomonas fluorescens* (Chapalain et al., 2009). Interestingly, PK 11195 was shown to increase adhesion and biofilm formation activities while decreasing the cytotoxic (apoptotic) effect of *Pseudomonas fluorescens* MF37 (Chapalain et al., 2009). These observations are consistent with the fact that the growth of *Pseudomonas* in biofilms or aggregates is associated with a decrease in virulence (Staudinger et al., 2014), and this supports the hypothesis that, as in *Pseudomonas syringae*, TSPO is involved in virulence expression.

In the three studied *Pseudomonas syringae* strains, the STRING analysis predicted that TSPO interacts with the chaperone subunit, GroL (Figure 3), a protein that belongs to the heat shock sigma factor RpoH regulon. Since RpoH is involved in the response to stresses leading to misfolded or aggregated proteins, it suggests that TSPO may be linked to this type of general stress

response. Interestingly, such functions have been proposed in plant TSPOs (Balsemão-Pires et al., 2011). Furthermore, in addition to their well-documented function as molecular chaperones, GroEL proteins are increasingly recognized as exhibiting surprising additional so-called “moonlighting” functions (Henderson et al., 2013). Perhaps the most astonishing feature of members of the GroEL family is that they can demonstrate toxic activities against eukaryotes (Kupper et al., 2014).

Membrane and cell wall biogenesis and/or integrity

TSPO was also predicted to interact with three proteins involved in membrane and cell wall biogenesis, including a putative nucleoside-diphosphate-sugar epimerase Pfl01_0720, the UDP-N-acetylmuramate-alanine ligase MurC, and OprF (Figure 3). OprF is the major OM porin of members belonging to the *Pseudomonas* genus, which is homologous to the mitochondrial VDAC. Functional links between OprF and TSPO have been demonstrated in *Pseudomonas fluorescens* MF37 (Chapalain et al., 2009). Notably, in addition to its structural role in anchoring the OM to the peptidoglycane layer, OprF has been shown to be required for full virulence expression in the human pathogen *Pseudomonas aeruginosa* (Fito-Boncompagni et al., 2011). OprF is also a receptor for human IFN γ , allowing the bacteria to sense the host's immune state (Wu et al., 2005). Since all of the *Pseudomonas* are not pathogens, it has further been proposed that this protein could act as a more general environmental sensor (Wagner et al., 2006). The interaction between TSPO and OprF suggests that they could participate in a common sensing/transducing network. This hypothesis is also supported by the observation of a possible functional link between TSPO and the adenine phosphoribosyl transferase (Apt, Figure 3), which enables the transfer of a phosphoribosyl group on adenine, thus producing AMP (Becerra and Lazcano, 1998). Interestingly, in mammals, TSPO is possibly associated with an ANT, which is localized in the inner membrane of mitochondria, and it plays an essential role in transporting ADP into the mitochondrial matrix and ATP out from the matrix for cell utilization (Leung et al., 2013). As such, we cannot exclude that as in eukaryotes, bacterial TSPO should form a ternary complex with an adenine-associated substrate translocating protein.

Signaling and signal transduction

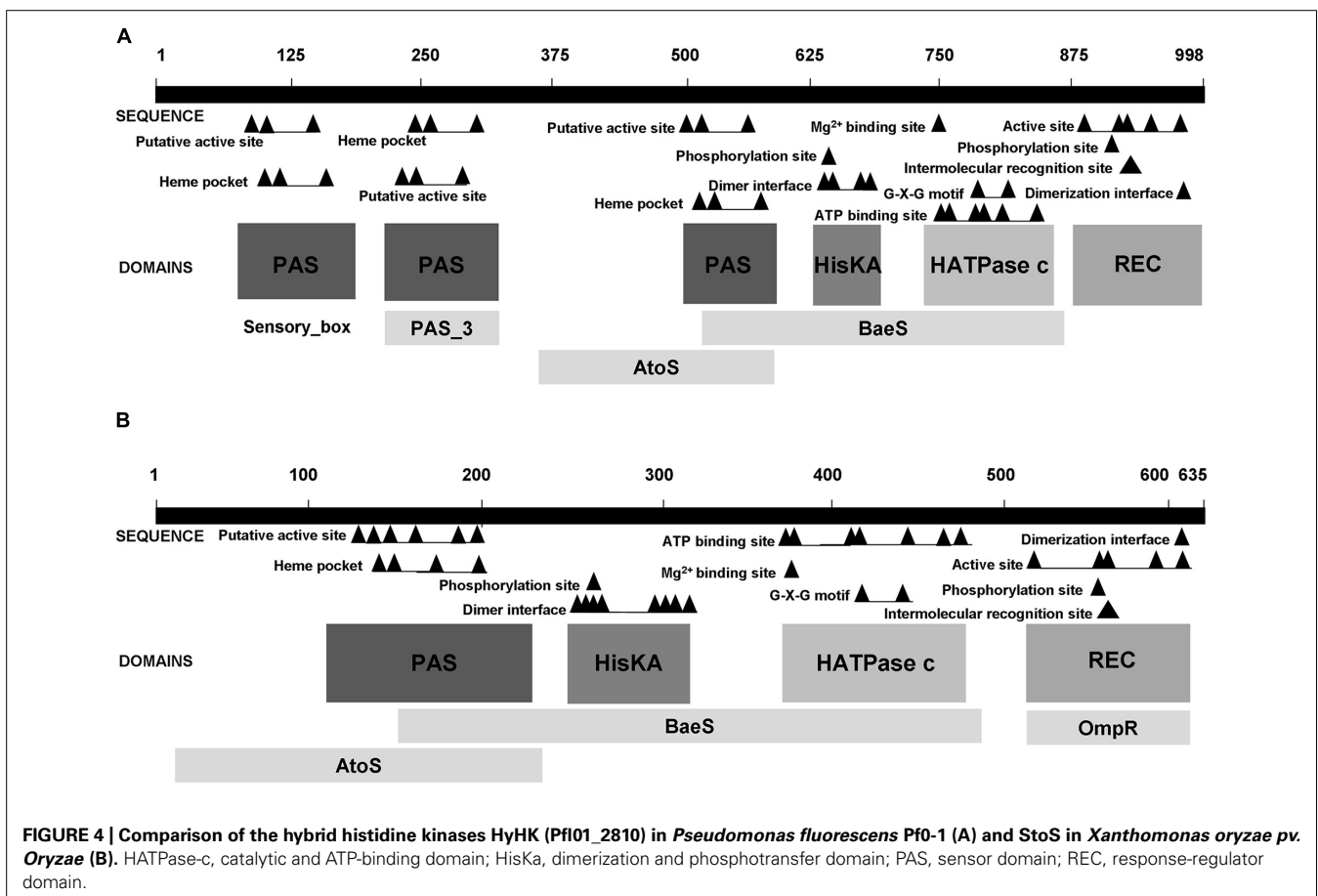
As mentioned in Section “Functions”, TSPO has been shown to be involved in gene regulation, noticeably in *R. sphaeroides*, in which TSPO has been proposed as an oxygen sensor (Yeliseev et al., 1997), and in *Sinorhizobium meliloti* (Davey and de Bruijn, 2000). In the latter, TSPO is involved in regulating the expression of the nutrient deprivation induced (*ndi*) locus. In this bacterium, the two component sensor FixL, has been shown to be required for full induction of the *ndi* locus expression. Interestingly, these authors demonstrate that TspO appears to be epistatic to the histidine kinase (HK) FixL, since no expression is observed in the *tspO* mutant, even though FixL is still present (Davey and de Bruijn, 2000). Using the STRING algorithm to investigate the putative interactions of TSPO in *Pseudomonas fluorescens* Pf0-1, we observed a potential link between TSPO and a hybrid HK (HyHK, Pfl0-1_2810; Figure 3). Moreover, in this bacterium, the two genes

appear to be located in the same operonic structure¹, suggesting the presence of genetic and functional links. Two component signal transduction systems generally consist of a sensor HK and a response regulator (RR) that contains a receiver or response regulator domain (REC). Hybrid-type HKs (HyHKs) comprise a HK with a receiver domain within one molecule (Stock et al., 2000). Interestingly, the *Pseudomonas fluorescens* Pf0-1 HyHK is predicted to possess six functional domains, among which three PAS domains (Figure 4). In the bacterial kingdom, PAS domains are commonly positioned at the amino terminus of signaling proteins such as sensor HKs, and tandem and multiple PAS domains are common in individual proteins: about one third of PAS proteins contain two or more PAS domains (Henry and Crosson, 2011). Interestingly, PAS ligand binding either functions as a primary cue to initiate a cellular signaling response or provides the domain with the capacity to respond to secondary physical or chemical signals such as gas molecules, redox potential, or photons (Henry and Crosson, 2011). Noticeably, many PAS-domain proteins detect their signal by way of an associated cofactor such as heme (Henry and Crosson, 2011), and this may be the case for the *Pseudomonas fluorescens* Pf0-1 HyHK since a heme pocket has been predicted in each of the three PAS domains (Figure 4). Interestingly, the *Pseudomonas fluorescens* Pf0-1 HyHK shows some domain similarities with the rice pathogen *Xanthomonas oryzae* pv. *Oryzae*, HyHK StoS (i.e., the four C-terminal domains), as

it includes a PAS domain with a heme pocket involving StoS as an oxygen sensor, a HisKa dimerization and phosphotransfer domain with an Mg²⁺ binding site, a HATPase energizing domain, and a receiver REC domain (Figure 4). Interestingly, StoS is activated by sensing low-oxygen concentrations, and it is involved in stress tolerance and virulence (Ikawa et al., 2014). As shown on Figure 4, the *Pseudomonas fluorescens* Pf0-1 HyHK, -as well as StoS-, shows domain homology with the BaeS HK, and to a lesser extent to the AtoS HK (Figure 4). In *E. coli*, the two component system (TCS) AtoSC has been involved in modulating diverse fundamental cellular processes such as short-chain fatty acid catabolism, poly-(R)-3-hydroxybutyrate biosynthesis and chemotaxis (Kyriakidis and Tiligada, 2009). In *E. coli*, the BaeSR TCS is involved in response to severe envelope stress like spheroplasting (Raffa and Raivio, 2002). Recently, it has been shown that, in *Salmonella enterica* serovar *Typhimurium*, BaeSR is required for expression of *sodA* and possibly of *sodB*, encoding two major SODs that are involved in ROS detoxification (Guerrero et al., 2013).

THE CASE OF *Pseudomonas aeruginosa*

Dealing with *Pseudomonas* without mentioning its best known representative species, *Pseudomonas aeruginosa*, should appear incomplete, but its absence all throughout this review was only motivated by the lack of the *tspo* analog in the genome of



Pseudomonas aeruginosa. Moreover, all attempts to identify even a truncated or partly deleted *tspo* sequence in *Pseudomonas aeruginosa* sequenced genomes were failures (Chapalain et al., 2009; Fan et al., 2012). This observation could appear as a simple illustration of the highly variable expression of bacterial TSPO. However, the situation should be more complex. Indeed, it has been shown that benzodiazepines improve the recovery of animals in the *Pseudomonas aeruginosa*-infected burned mouse model (Dugan et al., 2010), and a detailed analysis of the data indicates that the effect of benzodiazepines on the healing process is not due to direct stimulation of cutaneous cicatrization (Egydio et al., 2012). It is likely that there is no direct effect of benzodiazepines on *Pseudomonas aeruginosa* but the effects seen is due to the modulatory effect of benzodiazepines on the immune system (Egydio et al., 2012). This is similar to the case of epinephrine, known since 1930 to have a major impact on bacterial infections but whose direct effect on bacteria was only recognized by the end of the 20th century (Lyte and Ernst, 1992) – well before its bacterial sensor was identified (Clarke et al., 2006; Hughes and Sperandio, 2008). In fact, although *Pseudomonas aeruginosa* does not express TSPO, its large genome encodes many sensors (receptors) and transporters and different porins, including OprF. In addition, other studies indicated that benzodiazepine analogs can exhibit *in vitro* direct antibacterial activities on *Pseudomonas aeruginosa* (Sharma et al., 2013), suggesting that *Pseudomonas aeruginosa* can sense benzodiazepines. Considering these findings, we cannot exclude the possibility that a sensor protein showing functional homologies with TSPO is expressed in *Pseudomonas aeruginosa*.

Interestingly, the budding yeast *Saccharomyces cerevisiae* is known to lack TSPO, which may be due to the loss of almost 90% of duplicated genes in this organism (Kellis et al., 2004). This characteristic makes *Saccharomyces cerevisiae* well-suited for heteroexpression studies and an ideal model system for studying the biochemical and pharmacological properties of TSPO (Riond et al., 1991). However, the lack of TSPO in *Saccharomyces cerevisiae* also shows that TSPO is not essential for yeast viability and therefore other pathways could perform the functions attributed to the TSPO in other organisms.

CONCLUSION

TSPO is a protein whose typical five trans-membrane helix structure has been remarkably preserved along the evolutionary process, but whose localization and functions evolved from α - and γ -proteobacteria to eukaryotes. As a membrane protein, TSPO may be involved in redox and iron homeostasis, and virulence expression as its regulatory networks could be intimately intermingled. As is the case with mitochondria, in *Pseudomonas*, bacterial TSPO could also take part in signal transduction and in establishing membrane integrity. As a result, TSPO appears to be a vital protein in fluorescent *Pseudomonas*.

AUTHOR CONTRIBUTIONS

Charlène Leneveu-Jenvrin, Nathalie Connil, Emeline Bouffartiges, Vassilios Papadopoulos, Marc G. J. Feuilleley, Sylvie Chevalier wrote the manuscript and performed the *in silico* analysis.

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-Article 9-



Expression of the translocator protein (TSPO) from *Pseudomonas fluorescens* Pf0-1 requires the stress regulatory sigma factors AlgU and RpoH

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Expression of the translocator protein (TSPO) from *Pseudomonas fluorescens* Pf0-1 requires the stress regulatory sigma factors AlgU and RpoH

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The translocator protein (TSPO), previously designated as peripheral-type benzodiazepine receptor, is an evolutionary conserved protein that is found in many Eukarya, Archae, and Bacteria, in which it plays several important functions including for example membrane biogenesis, signaling, and stress response. A *tspo* homolog gene has been identified in several members of the *Pseudomonas* genus, among which the soil bacterium *P. fluorescens* Pf0-1. In this bacterium, the *tspo* gene is located in the vicinity of a putative hybrid histidine kinase-encoding gene. Since *tspo* has been involved in water stress related response in plants, we explored the effects of hyperosmolarity and temperature on *P. fluorescens* Pf0-1 *tspo* expression using a strategy based on *lux*-reporter fusions. We show that the two genes Pf101_2810 and *tspo* are co-transcribed forming a transcription unit. The expression of this operon is growth phase-dependent and is increased in response to high concentrations of NaCl, sucrose and to a D-cycloserine treatment, which are conditions leading to activity of the major cell wall stress responsive extracytoplasmic sigma factor AlgU. Interestingly, the promoter region activity is strongly lowered in a *P. aeruginosa algU* mutant, suggesting that AlgU may be involved at least partly in the molecular mechanism leading to Pf101_2810-*tspo* expression. *In silico* analysis of this promoter region failed to detect an AlgU consensus binding site; however, a putative binding site for the heat shock response RpoH sigma factor was detected. Accordingly, the promoter activity of the region containing this sequence is increased in response to high growth temperature and slightly lowered in a *P. aeruginosa rpoH* mutant strain. Taken together, our data suggest that *P. fluorescens tspo* gene may belong at least partly to the cell wall stress response.

Keywords: TSPO, AlgU, *rpoH*, osmolarity, temperature

Introduction

Tryptophan-rich sensory protein/peripheral-type benzodiazepine receptor (TspO/MBR) domain-containing proteins also called translocator proteins (TSPOs) are membrane anchored proteins that have already been described in several bacteria, Archae, and Eukarya (Lacapere and Papadopoulos, 2003; Papadopoulos et al., 2006; Fan et al., 2012; Leneveu-Jenvrin et al., 2014).

The best characterized member of this family is the mammalian 18-kDa TSPO protein, which is essentially located in the mitochondrial outer membrane associated with the voltage-dependent anion channel (VDAC). It has been involved in many fundamental physiological functions in mammals, including cell growth and proliferation, immunomodulation, apoptosis, and adaptation to oxidative stress (Papadopoulos et al., 2006; Guo et al., 2015), as well as in various activities related to mitochondrial physiology, including cholesterol import and steroid hormone biosynthesis (Papadopoulos et al., 1997; Miller and Bose, 2011; Issop et al., 2013). In mammals, TSPO is expressed in almost every tissue type, but is particularly enriched in steroidogenic and some cancer cells (Hardwick et al., 1999; Galiegue et al., 2003). TSPO expression dysregulation has been correlated to several diseases, including cancer (Batarseh and Papadopoulos, 2010), neuronal damage, neurodegeneration, and inflammation (Harberts et al., 2013; Dickens et al., 2014). In addition, a second mammalian TSPO isoform that is involved in erythropoiesis has been described (Fan et al., 2009). Overall, considering these important functions and perspectives in mammals, TSPO has been the focus of multiple studies during these last 30 years. However, the precise physiological function of TSPO in mammals remains a controversial, elusive, and currently intensively debated question (Batoko et al., 2015; Gut et al., 2015; Li et al., 2015; Milenkovic et al., 2015; Papadopoulos et al., 2015; Selvaraj and Stocco, 2015). For example, while TSPO was shown to bind many ligands including cholesterol (Li and Papadopoulos, 1998) and porphyrins (Verma et al., 1987), its function as cholesterol or porphyrin translocator is yet still unclear (Morohaku et al., 2013; Batoko et al., 2015; Gut et al., 2015; Li et al., 2015; Tu et al., 2015). Very recently, it has been proposed that TSPOs may be involved at least partly in complex homeostasis signaling mechanisms, related in particular to oxidative stress (Batoko et al., 2015).

TSPO-related proteins were also identified in plants (Corsi et al., 2004; Lindemann et al., 2004; Frank et al., 2007). In the moss *Physcomitrella patens*, three genes encode *tspo* homologs. One of these, PpTSPO1, is up-regulated by abiotic stress at the transcriptional level, and a knockout mutant of this gene led to increased sensitivity to salt stress (Frank et al., 2007). In angiosperm species such as *Arabidopsis thaliana*, a single gene encodes a TSPO-related protein (AtTSPO). AtTSPO is transiently expressed in dry seeds and can be induced in vegetative tissues by osmotic and salt stresses or by abscisic acid (ABA) treatments, suggesting that AtTSPO is specifically induced by water-related stress (Guillaumot et al., 2009a,b; Balsemão-Pires et al., 2011). Constitutive expression of this protein has been found to be detrimental to plant cells and seems to increase their sensitivity to ABA, suggesting that AtTSPO may be required transiently in an ABA-dependent stress response (Guillaumot et al., 2009a,b).

Bacterial TSPO appears to be widely distributed in eubacteria since a *tspo* homolog gene has been predicted in 97 different eubacterial species or strains encompassing most of the different taxonomic groups (Chapalain et al., 2009). The best characterized member of the *tspo* family is found in the proteobacterium *Rhodobacter sphaeroides* (RsTSPO). RsTSPO is localized in the

bacterial outer membrane and its expression is induced by oxygen (Yeliseev and Kaplan, 1999). Under conditions of high oxygen, TSPO was shown to function as a negative transcriptional regulator of genes involved in photo-pigment biosynthesis, likely controlling the efflux of an intermediate in the heme biosynthesis pathway (Yeliseev and Kaplan, 2000; Zeng and Kaplan, 2001). A similar mechanism was proposed for the regulation of a nutrient deprivation-induced (*ndi*) locus in the endosymbiotic soil bacteria *Sinorhizobium meliloti* (Davey and de Bruijn, 2000).

Interestingly, a *tspo* homolog gene has been identified in several members of the *Pseudomonas* genus, among which *P. fluorescens* Pf0-1 (Chapalain et al., 2009; Leneveu-Jenvrin et al., 2014). *P. fluorescens* Pf0-1 is a common psychrotrophic Gram-negative inhabitant of soil and rhizosphere, and in its genome the *tspo* gene is located in the vicinity of a putative hybrid histidine kinase-encoding gene (Leneveu-Jenvrin et al., 2014). In this strain, both genes are predicted to form an operonic structure (Winsor et al., 2011). This study aims at giving new insights into *tspo* expression of *P. fluorescens* Pf0-1 using a strategy based on *lux*-reporter fusions. Since *tspo* has been involved in water stress related-response in plants, we explored the effects of such stresses on *P. fluorescens* Pf0-1 *tspo* expression.

Materials and Methods

Bacterial Strains and Culture Conditions

The strains used are listed in **Table 1**. *P. fluorescens* strains were grown at 17, 28, or 32°C on a rotary shaker (180 rpm) in Luria-Bertani (LB) broth supplemented or not with additional NaCl 171 (LB171N) or 342 (LB342N) mM, sucrose 342 (342S) or 684 (LB684S) mM, or D-cycloserin 50 $\mu\text{g ml}^{-1}$. For each condition, a pre-culture was inoculated at an initial optical density at 600 nm (OD_{600}) of 0.08 in LB medium. Stationary phase cells grown in LB medium were diluted at 1/100 (v/v) in LB or in modified LB, and their growth was followed in covered white 96-well OptiPlates with a flat transparent bottom (BD Falcon, San Jose, CA, USA), unless otherwise specified. Bacterial growth was monitored throughout time using a multimode plate reader (Flex-Xenius XM; SAFAS, Monaco). When required (for plasmid maintenance), *P. fluorescens* Pf0-1 or *P. aeruginosa* PA01 was grown at 28 or 37°C, respectively, in LB liquid cultures in the presence of Gm (50 $\mu\text{g ml}^{-1}$), or on LB agar (1.5%) plates containing Gm (50 $\mu\text{g ml}^{-1}$).

Construction of Transcriptional Fusions

To monitor *tspo* transcription, *tspo* and *Pfl01_2810*-*tspo* promoter regions were fused to the promoter-less *luxCDABE* cassette in the replicative low-copy-number pAB133 vector (Gm^r) (Bazire et al., 2005). The promoter regions of interest are located in **Figures 1A** and **5A**, relatively to the *tspo* and *Pfl01_2810* genes. The primer sequences, which were used for PCR amplifications, are shown in **Table 2**. The *SacI*-*SpeI*-digested PCR products were inserted into pAB133, yielding pTSPO, pHK-TSPO, pHK-TSPO-227, pHK-TSPO-157, and pHK-TSPO-88. The inserts were verified by DNA sequencing (Beckman Coulter Genomics).

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

Strain or plasmid	Characteristics	Reference
Strains		
<i>Pseudomonas fluorescens</i>		
Pf0-1	Wild type	Silby et al., 2009
<i>P. aeruginosa</i>		
PAO1	Wild type	Woodruff and Hancock, 1989
PAOU	PAO1 Δ algU	Bazire et al., 2010
<i>rpoH</i>	Transposon mutant <i>rpoH::IS;phoA/hah-Tc^r</i>	University of Washington mutant library (Jacobs et al., 2003)
Plasmids		
pAB133	Gm ^r ; pBBR1MCS-5-based cloning vector containing the promoterless <i>luxCDABE</i> operon.	Bazire et al., 2005
pTSPO	Gm ^r ; pAB133-based plasmid with the 253-bp <i>tspo</i> upstream fragment at the <i>SacI</i> and <i>SpeI</i> sites.	This study
pHK-TSPO	Gm ^r ; pAB133-based plasmid with the 227-bp Pfl01_2810- <i>tspo</i> upstream fragment at the <i>SacI</i> and <i>SpeI</i> sites	This study
pHK-TSPO-88	Gm ^r ; pAB133-based plasmid with the 89-bp Pfl01_2810- <i>tspo</i> upstream fragment at the <i>SacI</i> and <i>SpeI</i> sites.	This study
pHK-TSPO-157	Gm ^r ; pAB133-based plasmid with the 70-bp Pfl01_2810- <i>tspo</i> upstream fragment at the <i>SacI</i> and <i>SpeI</i> sites	This study
pHK-TSPO-227	Gm ^r ; pAB133-based plasmid with the 69-bp Pfl01_2810- <i>tspo</i> upstream fragment at the <i>SacI</i> and <i>SpeI</i> sites	This study

Bioluminescence Assays

Pseudomonas fluorescens or *P. aeruginosa* strains containing pAB133-derived plasmids were grown in covered, white 96-well OptiPlates with a flat transparent bottom (BD Falcon, San Jose, CA, USA). Bioluminescence and absorbance were simultaneously measured throughout bacterial growth using a multimode plate reader (Flex-Xenius XM; SAFAS, Monaco). The bioluminescence values (in relative light units [RLU] \cdot 0.5 s⁻¹) were divided by the absorbance values at 600 nm, yielding to relative bioluminescence values (in RLU \cdot 0.5 s⁻¹ \cdot A₆₀₀⁻¹). The relative luminescence (RL) values of the negative-control strain *P. fluorescens* or *P. aeruginosa* harboring the empty vector pAB133 were subtracted from those of the studied *Pfl01_2810-tspo* promoter regions, as previously described (Bouffartigues et al., 2012). Each set of experiments was performed at least three times.

Total RNAs Extraction

Total RNA extraction was achieved by the hot acid-phenol method. Briefly, cells were lysed and RNAs were extracted three times with an equal volume of acidic hot phenol and once with chloroform. After ethanol precipitation, RNAs were air dried and dissolved in water. Contaminating DNA was removed from total

RNA by using 10 U of RNase-free DnaseI (New England BioLabs France) in a 50 μ l mixture containing 6.25 mM MgCl₂ and approximately 3 μ g μ l⁻¹ of total RNA. The reaction mixture was incubated 30 min at 37°C, and the DNase I was then inactivated by adding 1 μ l of 0.5 M EDTA to the mixture and incubating the mixture for 10 min at 65°C. The concentration was determined by measuring the absorbance at 260 nm. The quality of the RNA was then checked on a 2% agarose gel prior to use. Each RNA extraction was tested by PCR for the absence of contaminating DNA prior reverse transcription assays, and was performed in triplicate.

RT-PCR/qRT-PCR Experiments

The primers used for reverse transcription-PCR (RT-PCR) or quantitative reverse transcription-PCR (qRT-PCR) are given in **Table 2**. Reverse transcription was used to assay co-transcription of genes belonging to the same operonic structure. Briefly, 10 ng of purified and controlled total RNAs were subjected to a one step RT-PCR assay according to the manufacturer instructions (Life Science, Roche, USA), before gel electrophoresis. qRT-PCR assays were achieved according to Gicquel et al. (2013), 16S rRNA was used as an endogenous control for qRT-PCR experiments (Gicquel et al., 2013), and the standard deviations were lower than 0.15 threshold cycle (CT). The relative quantifications were obtained as previously described, using the comparative CT ($-2^{\Delta\Delta CT}$) method (Livak and Schmittgen, 2001).

Statistical Analyses

All experiments are the mean \pm SEM of a minimum of three independent experiments. Significances of differences between mean values were assessed using the Student's *t*-test with significance set as **P* < 0.05, ***P* < 0.01.

Bioinformatics

MEME search was achieved according to Brown et al. (2013).

Results

Genomic Context and Transcriptional Analysis of *P. fluorescens tspo* and Pfl01_2810 Encoding a Putative Histidine Kinase

A previous study based on the comparative genome organization of *tspo* and of its flanking genes, revealed that the *tspo* environment was not conserved among bacteria (Chapalain et al., 2009). A *tspo* homolog gene has been predicted in only 7 out of the 48 fully sequenced genomes of *Pseudomonas* (Winsor et al., 2011), among which three are *P. syringae* (pv. *phaseolicola* 1448A, pv. *syringae* B728a, and pv. *tomato* DC3000), and three *P. fluorescens* (strains SBW25, Pf0-1, A506), and in *P. poae* (strain RE*1-1-14). The genomic environment of *tspo* is not conserved among these seven strains, although some degree of conservation exists in each species. For example, in two of the three studied *P. fluorescens* strains, SBW25 and A506, *tspo* forms an operonic structure with an esterase-encoding gene that is involved in lipid metabolism (Winsor et al., 2011). This was, however, not the case for *P. fluorescens* strain Pf0-1. In the

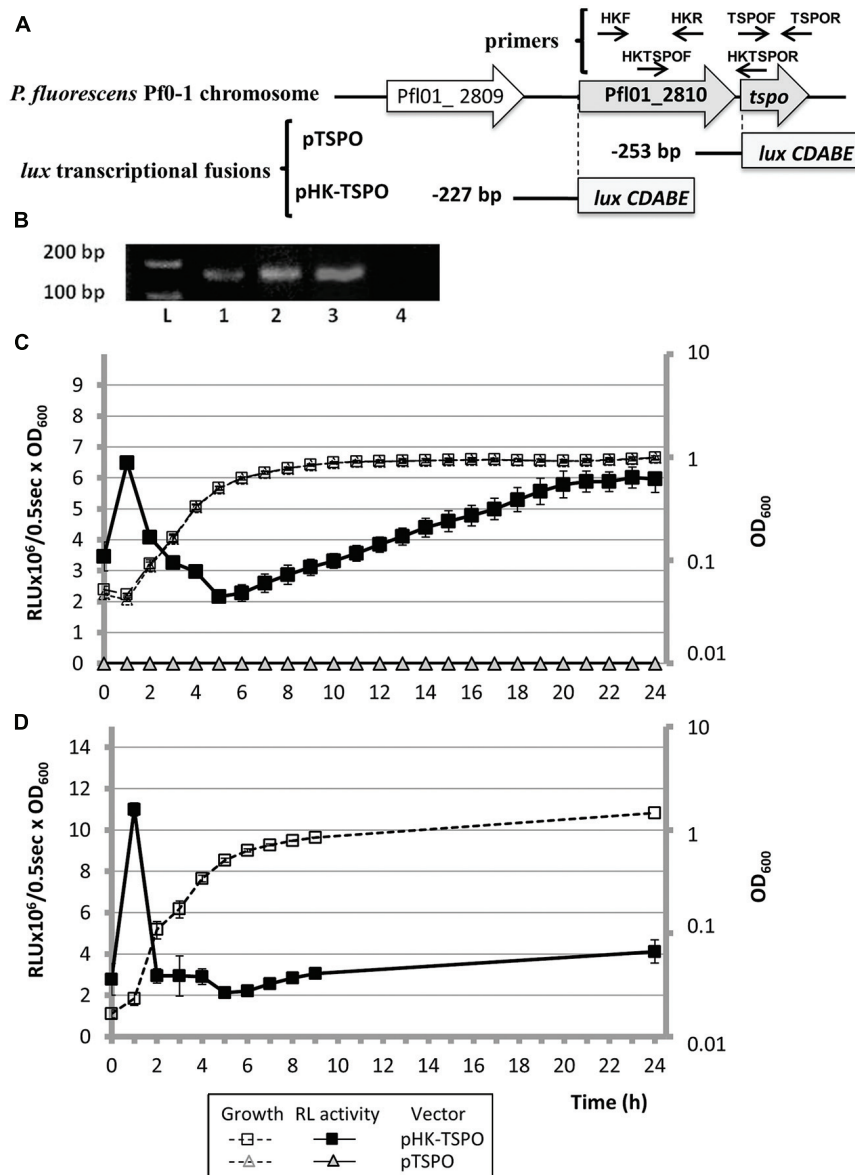
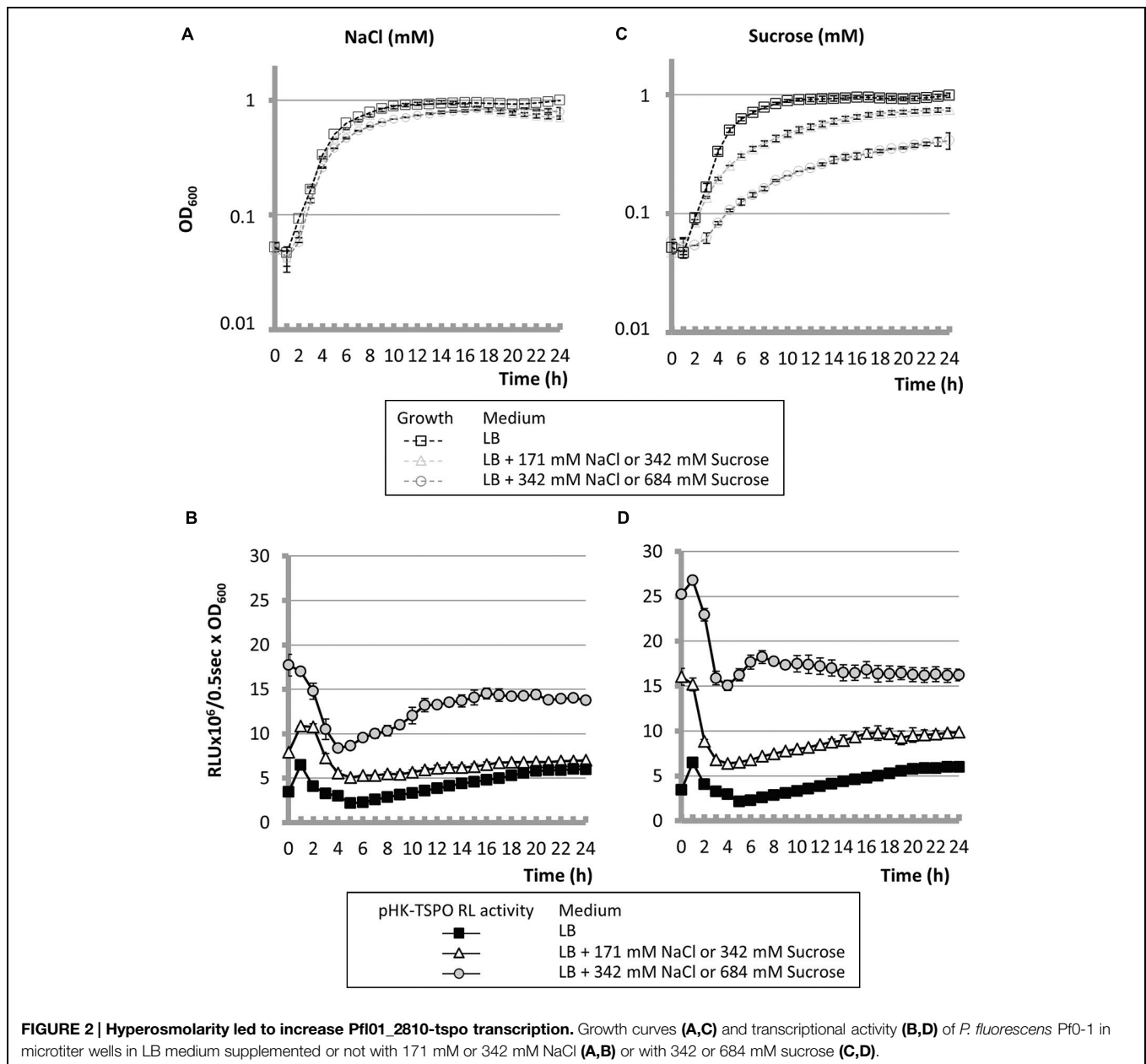


FIGURE 1 | The *tspo* gene (Pfi01_2811) of *Pseudomonas fluorescens* Pf0-1 forms an operonic structure with Pfi01_2810 that is expressed transiently in the bacterial growth course. (A) Schematic representation of the genomic environment of *tspo* and of transcriptional fusions pTSPO and pHK-TSPO. Gray bars represent the beginning of the *luxCDABE* reporter cassette of the promoterless pAB133 vector. The position of the studied promoter region is indicated relatively to the translational initiation start of each ORF. (B) Co-transcription of Pfi01_2810 and *tspo* by reverse transcription-PCR (RT-PCR) assay. RT-PCR was assayed on *tspo* (1), Pfi01_2810 (2) and on the putative operonic structure Pfi01_2810-*tspo* (3), using primers located into *tspo* (1) or Pfi01_2810 (2) ORFs, or into both *tspo* and Pfi01_2810 ORFs (3). RT-PCR achieved on total RNA did not lead to a PCR fragment (line 4, negative control). L: Ladder 1kb+ (Biorad®), primers sequences are given in **Table 2. (C) Growth curves in microtitre wells in Luria-Bertani (LB) medium at 28°C and relative luminescence (RL) activity of pTSPO and pHK-TSPO in *P. fluorescens* Pf0-1. (D) Growth curves in erlenmeyer in LB medium at 28°C and RL activity of pHK-TSPO in *P. fluorescens* Pf0-1.**

latter, a putative histidine kinase encoding gene (Pfi01_2810) was predicted 54 bp upstream of *tspo*. *In silico* predictions, based on the non-detection of a transcriptional termination signal, suggest that these two genes are included in an operonic structure (**Figure 1A**; Leneveu-Jenvrin et al., 2014). To get further insights into the genetic relations that may exist between the two genes, we investigated their co-transcription by RT-PCR experiments using primers HKF/R, TSPOF/R, HKTSPOF/R

designed on Pfi01_2810, or on *tspo*, or on both the two genes, respectively (**Table 2**; **Figure 1A**). A DNA fragment, reflecting the production of mRNA, was observed in each case (**Figure 1B**, lanes 1, 2, and 3), suggesting that the two genes can be co-transcribed in our conditions. A transcriptional fusion, in which a 253 bp fragment laying upstream the +1 translation start site of *tspo* was fused to the *luxCDABE* reporter system (**Figure 1A**, pTSPO), did not reveal any activity when bacteria



were grown in LB medium at 28°C (Figure 1C, full gray triangles), indicating the absence of promoter in this region. Taken together, our data suggest that Pfl01_2810 and *tspo* are co-transcribed in these conditions, suggesting not only genetic, but also functional links between these two genes in *P. fluorescens* Pf0-1.

Pattern of *tspo* Transcription during the Growth Course of *P. fluorescens* Pf0-1

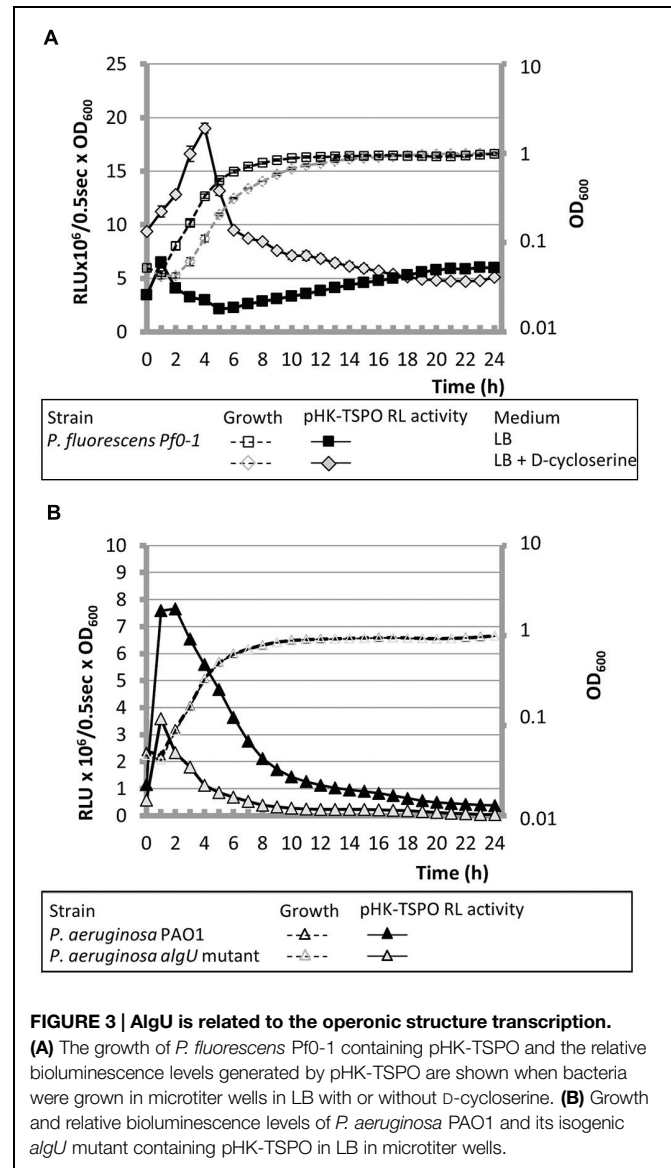
To further monitor the transcription of the Pfl01_2810-*tspo* operonic structure, we constructed the pHK-TSPO reporter fusion that contains the 227 bp fragment laying upstream the +1 translational start site of Pfl01_2810 fused to the *luxCDABE* cassette (Figure 1A). The activity of pHK-TSPO was followed in

LB medium at *P. fluorescens* Pf0-1 optimal growth temperature (28°C) for 24 h. The RL activity resulting from pHK-TSPO was increased by about 1.7-fold in the early exponential phase, and then it decreased rapidly further in the exponential phase (Figure 1C, black squares). After 6 h of growth, corresponding to the transition between the late exponential and the entry into the stationary growth phases, the promoter region activity increased slowly but continuously reaching a maximal value of almost 6×10^6 RLU/0.5 s/OD₆₀₀ after 24 h of growth. These data suggest that Pfl01_2810-*tspo* is expressed in a two-step process, i.e., transiently during the early exponential phase, and then, after the transition from exponential to stationary phases, slowly increasing throughout the stationary growth phase. Since the *lux* reporter system requires large amounts of energy and

oxygen, we questioned if these growth circumstances might be responsible for the large decrease in promoter activity during the course of growth. We therefore further tested the promoter activity in cells grown in Erlenmeyer flasks (10 ml of culture in 100-ml flasks) under vigorous shaking conditions (180 rpm). When considering the exponential growth phase, the growth and expression patterns were similar in microtiter plates and in Erlenmeyer flasks (Figures 1C,D, respectively), showing that the promoter activity during this phase was not due to the specific growth condition imposed by using microtiter plates, but rather reflected decreased transcription of *tspo* during *P. fluorescens* growth, regardless of the aeration status. By contrast, the increase of the promoter activity during the stationary growth phase could not be reproduced in Erlenmeyer flasks, suggesting that this effect would be linked to the cultural conditions. We thus choose to focus our study on the expression pattern of the operonic structure during the exponential growth phase. Taken together, our data indicate that the peak of activity of pHK-TSPo was growth phase dependent and transient, suggesting that the expression of Pfl01_2810 – *tspo* was highly regulated.

High Salinity and Hyperosmolarity Increase *tspo* Expression

In the plant *A. thaliana*, expression of AtTSPo is induced in vegetative tissues by high concentrations of NaCl suggesting that AtTSPo is specifically induced by water-related stress (Guillaumot et al., 2009a,b; Balsemão-Pires et al., 2011). Pfl01_2810 – *tspo* transcription was thus assayed in *P. fluorescens* Pf0-1 grown in LB supplemented with 171 (LB171N) or 342 mM NaCl (LB342N). As shown on Figure 2A, the bacterial growth was not deeply affected by the extra supplementation of the LB medium with NaCl. The maximal activities of the promoter region were about 1.7- and 2.9-fold higher during the early exponential growth phase when bacteria were cultured in LB171N or LB342N, respectively, comparatively to LB (Figure 2B). The maximal activity reached during the stationary growth phase was similar when bacteria were grown in LB or in LB supplemented with 171 mM of NaCl (LB171N). However, we observed a 2.4-fold increase when we compared the promoter activity in bacteria grown in LB342N and LB at this physiological stage. Taken together, our data suggest that high salinity increased Pfl01_2810 – *tspo* operonic structure expression, as it was the case for AtTSPo (Guillaumot et al., 2009a,b). To assay if salinity or osmolarity was the cause of this increase, we then supplemented LB with sucrose at the same osmolarity as NaCl. LB171N contains 171 mM additional NaCl, i.e., 342 mM osmolytes, since it can dissociate into two ionic species. Addition of 342 and 684 mM sucrose to LB medium led to generate LB342S and LB684S, which have the same osmolarity as LB171N and LB342N, respectively. As shown on Figure 2C, increasing the concentration of sucrose led to an increased doubling time, indicating that the presence of sucrose impaired the growth of *P. fluorescens* Pf0-1 (doubling times were 1.5- and 2.3-fold increased in LB342S and 684S compared to LB medium, respectively). The bioluminescence patterns in both media were similar to that observed in LB (Figure 2D). The maximal activities of the promoter region



were 2.6- and 4.3-fold higher during the early exponential growth phase when bacteria were cultured in LB342S or LB684S, respectively, in comparison to LB. The maximal activity reached during the stationary growth phase was increased 1.8- and 2.9-fold in LB342S and LB684S compared to LB (Figure 2D). Taken together, our data show that transcription of Pfl01_2810 – *tspo* was increased not only in response to high salinity like AtTSPo (Guillaumot et al., 2009a,b), but also in response to hyperosmolarity.

AlgU is Involved in *tspo* Expression

In members of the *Pseudomonas* genus, the response to hyperosmolarity has been shown to be triggered by the master extracytoplasmic function (ECF) sigma factor AlgU (Schneider-Keel et al., 2001). In *P. aeruginosa* mucoid strains, AlgU has been extensively studied due to its involvement in the mucoid conversion, leading to increasing the severity of cystic fibrosis

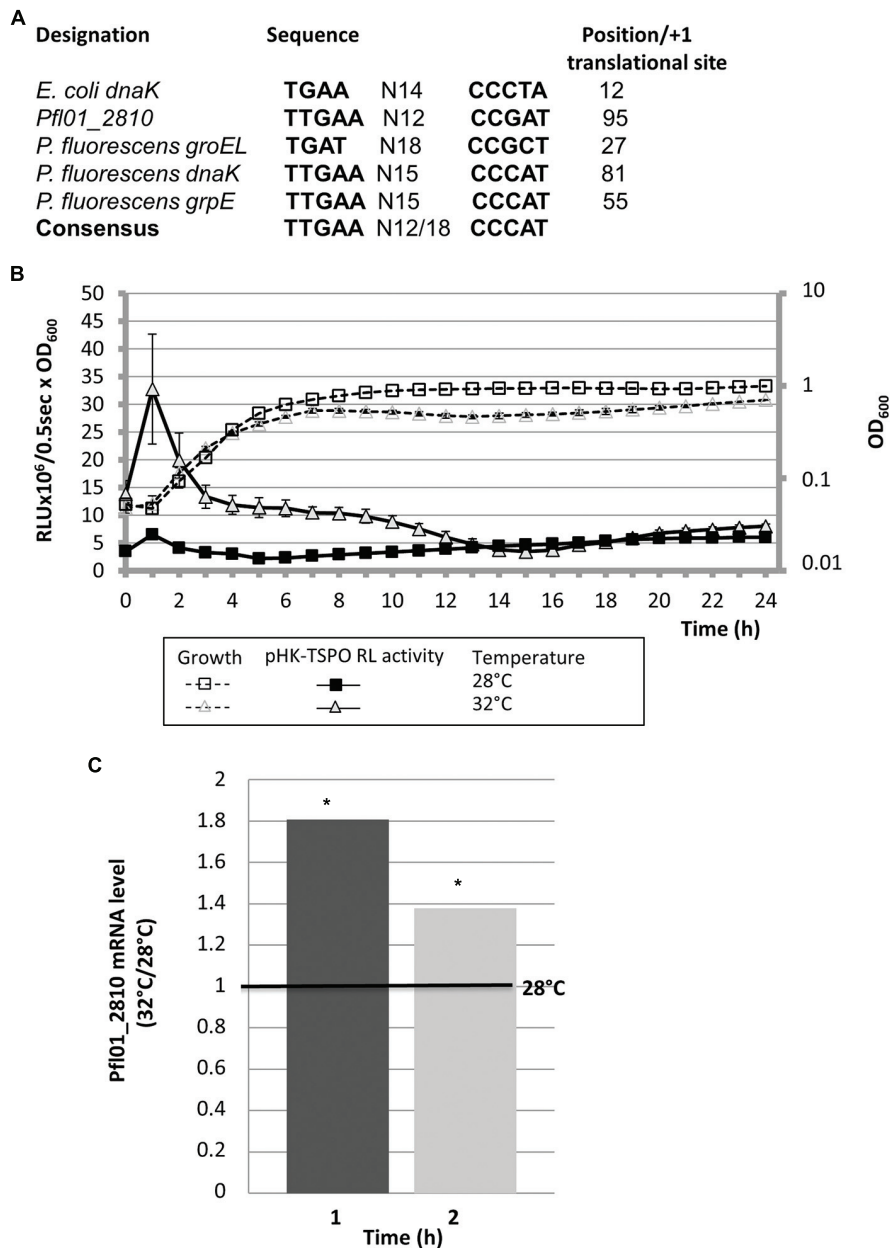


FIGURE 4 | A putative RpoH-consensus binding site is located in Pf01_2810-*tspo* promoter region: effect of temperature on growth and TSPO expression. (A) Sequence alignment of the putative RpoH binding sites identified by MEME in the regions located upstream of *dnaK* in *E. coli* and *groEL*, *dnaK*, *grpE*, and Pf01_2810 in *P. fluorescens* Pf0-1, relatively to the *E. coli* RpoH consensus sequence. Localization of this sequence is given relatively to the +1 translational start site. **(B)** Growth and transcriptional activity of *P. fluorescens* Pf0-1 containing pHK-TSPO reporter fusion in microtiter plates cultured at 28 or 32°C. **(C)** Relative expression of Pf01_2810 when bacteria were grown at 32°C compared to 28°C by qRT-PCR assays. Significances of differences between mean values were assessed using the Student's *t*-test with significance set as **P* < 0.05.

symptoms (Schurr et al., 1996). In non-mucoid strains, AlgU is the major cell wall stress response regulator (Wood et al., 2006). AlgU has been shown to be activated in response to sub-lethal concentrations of D-cycloserine antibiotic leading to peptidoglycan alterations in *P. aeruginosa* (Wood et al., 2006). In an attempt to give further insights into Pf01_2810 - *tspo* expression, 50 $\mu\text{g}\cdot\text{ml}^{-1}$ of D-cycloserine was added

to *P. fluorescens* Pf0-1 growth medium. At this sub-lethal concentration, the presence of the antibiotic slightly reduced the growth parameters of *P. fluorescens*, without affecting the final biomass (Figure 3A). The presence of D-cycloserine strongly increased the activity of pHK-TSPO during the exponential growth phase. However, the promoter region activity decreased rapidly, without showing an increase during the stationary

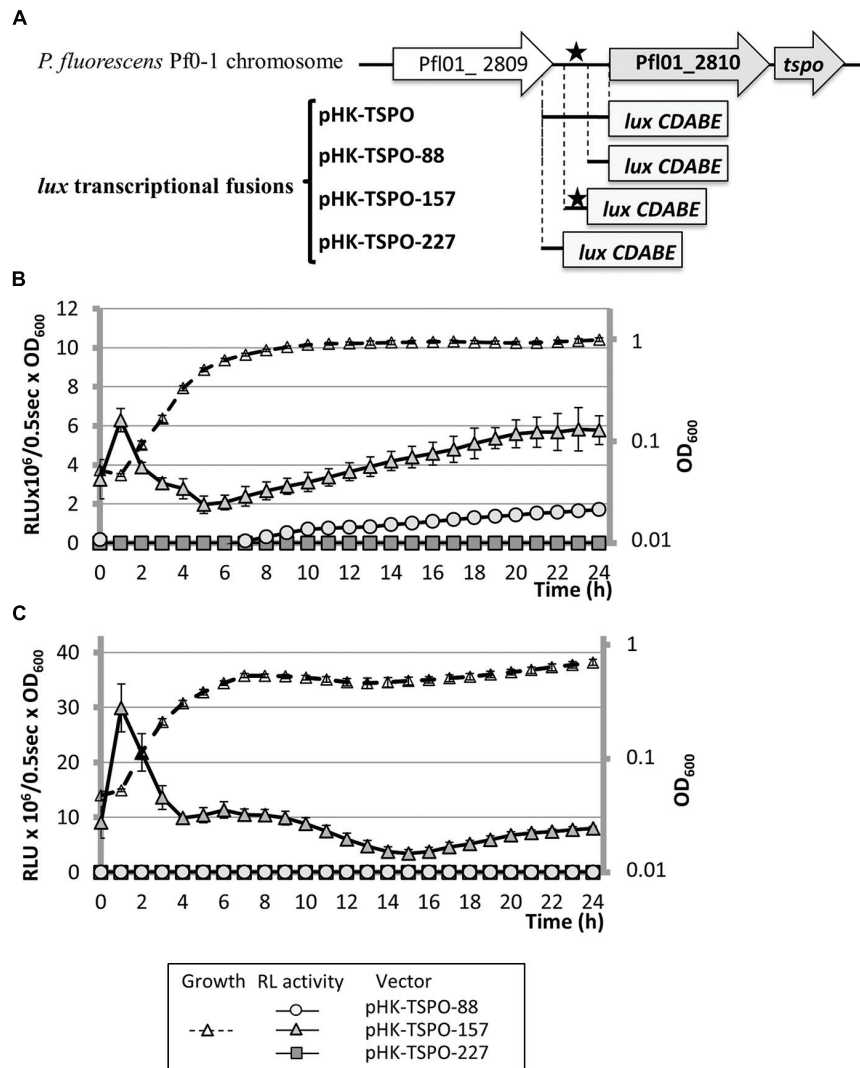


FIGURE 5 | The activity of the promoter region containing the putative RpoH binding site is increased in response to enhanced growth temperature.

(A) Schematic representation of the four transcriptional fusions. The position of the studied promoter region is indicated relatively to the translational initiation start of each ORF (+1). Putative RpoH binding sequence (black star). Gray bars represent the beginning of the *luxCDABE* reporter cassette of the promoterless pAB133 vector. (B,C) Growth in microtiter plates at 28°C (B) or 32°C (C), and transcriptional activity of *P. fluorescens* Pf0-1 containing pHK-TSPO-88, pHK-TSPO-157, and pHK-TSPO-227 plasmids.

growth phase, as it was observed after a hyperosmolar treatment. Taken together, these data suggest that the first peak could be linked to the activity of AlgU in our conditions, and that the increase observed during the stationary growth phase under hyperosmolar treatment could be AlgU-unrelated. To get further insight into the role of AlgU in the Pfl01_2810 – *tspo* transcription, the pHK-TSPO reporter fusion and the pAB133 empty vector were transferred into *P. aeruginosa* PAO1 and in its *algU* isogenic mutant strain (Bazire et al., 2010). As in the case of *P. fluorescens* Pf0-1, the promoter fusion activity increased during the early exponential growth phase of wild-type *P. aeruginosa*, but was clearly reduced by more than 2 fold in the *algU* mutant strain (Figure 3B). However, no further increase of the fusion activity was observed during the stationary phase in *P. aeruginosa* wild

type and *algU* mutant strains, suggesting that this effect might be specific to *P. fluorescens* Pf0-1 and/or to the microtiter growth conditions, as discussed above. Taken together, these data suggest an involvement of AlgU in the operonic structure transcription.

Expression of Pfl01_2810-*tspo* Operon is Regulated Partly through the Alternative Sigma Factor RpoH

A bioinformatic analysis of the 227 bp region lying upstream of Pfl01_2810-*tspo* using the MEME software (Brown et al., 2013), failed to identify an *algU* consensus binding site (data not shown), suggesting that the effect of AlgU on the expression of this promoter region may be indirect. However, a sequence that showed 90% of identity with the previously proposed binding

TABLE 2 | Primers used in this study.

Primer	Name	Sequence (5'–3')*
HKF		GCCCTGCTCAACCTGTGTAT
HKR		CCTAGCGGTTTGGTGGTAAA
TSPOF		AACAAGCCGAAATTCACACC
TSPOR		CACAGCAGGACGAGAATGAG
HKTSPOF		CGGGTCAGTGAATTGATGTG
HKTSPOR		GGTGTGAATTTTCGGCTTGT
F1	pTSPO	taataa <u>gagctc</u> AGCGCATGAGCATGATGTA
R1	pTSPO	taataa <u>actagt</u> TCCGACAGGTGCAGTCAAT
F2	pHK-TSPO	taataa <u>gagctc</u> ATGAACGGTGCACAACCTGG
R2	pHK-TSPO	taataa <u>actagt</u> TTCTTACTCCTTGGCCGC
F3	pHK-TSPO-227	taataa <u>gagctc</u> AGCGCATGAGCATGATGTAC
R3	pHK-TSPO-227	taataa <u>actagt</u> CGTTGCCAGAGTGCCTGT
F4	pHK-TSPO-157	taataa <u>gagctc</u> CGGCCAGGCATTGTTGG
R4	pHK-TSPO-157	taataa <u>actagt</u> GTCGTATCGGGCTAGCACT
F5	pHK-TSPO-88	taataa <u>gagctc</u> CTTGCTCCATCAGTAACTTAA
R5	pHK-TSPO-88	taataa <u>actagt</u> ACAGGTGCAGTCAATATCGG
16SF		CTGGTAGTCCACGCCGTAAC
16SR		CCAGGCCGTCAACTTAATGC
Pfl012810F		AGCTGATGCCGCTCTACGA
Pfl012810R		GTACGG-TCGCGGAGAATTTTC

*All primers used in this study were synthesized by Eurogentec and are based on *P. fluorescens* Pf0-1 genome sequence (<http://www.pseudomonas.com>). Nucleotides in lowercase are not complementary to the target sequence. Underlined nucleotides indicate restriction endonuclease sites inserted within primer sequences.

consensus sequence for the *E. coli* RpoH sigma factor ($\sigma 32$) was found 95 bp upstream the putative +1 translational start site of Pfl01_2810 (Cowing et al., 1985; **Figure 4A**). This consensus sequence was also detected upstream *P. fluorescens* Pf0-1 *dnaK*, *groEL*, or *grpE* genes (**Figure 4A**) encoding established direct RpoH targets in *P. aeruginosa* (Potvin et al., 2008) and/or in *E. coli* (Arsene et al., 2000). In these latter bacterial species, RpoH is responsible for the heat-shock response during the upshift of temperature from 30 to 42°C in the early exponential growth phase (Arsene et al., 2000). *P. fluorescens* Pf0-1 is a psychrotrophic strain that is able to grow at temperatures up to 32°C (Silby et al., 2009; Loper et al., 2012). We therefore tested the effect of growth at 32°C on the expression of the operonic structure Pfl01_2810-*tspo*. The promoter activity of the operonic structure was about fivefold increased during the exponential growth phase when bacteria were grown at 32°C compared to 28°C (**Figure 4B**). By contrast, no temperature-related modification could be observed during the stationary growth phase (**Figure 4B**). Pfl01_2810 mRNA expression level was further measured by qRT-PCR experiments on RNAs extracted from bacteria grown at 28 or 32°C. As shown on **Figure 4C**, an increase of the growth temperature resulted in significantly higher levels of expression (about 1.8- and 1.4-fold Pfl01_2810 expression after 1 and 2 h of growth,

respectively). Taken together, our data show that the activity of the promoter region laying upstream Pfl01_2810 – *tspo* is increased in response to an elevation of the growth temperature. The identification of an RpoH consensus binding site upstream of Pfl01_2810 is therefore in line with the increased activity of this promoter region at elevated growth temperature. To get further insights into the involvement of RpoH in the temperature-dependent increase of the promoter activity, the transcriptional fusions pHK-TSPO-227, pHK-TSPO-157, pHK-TSPO-88 were constructed, in which three fragments of the studied promoter region were fused to the *luxCDABE* reporter system (**Figure 5A**). Among these three fusions, only pHK-TSPO-157 contained the RpoH putative binding site (**Figure 5A**, black star). The promoter activity of each construct was then assayed when bacteria were grown at 28° (**Figure 5B**) and 32°C (**Figure 5C**). The fusion containing the RpoH-dependent promoter was active at both growth temperatures (**Figures 5B,C**, full triangles), at levels similar to the ones obtained with the large promoter region fusion construct pHK-TSPO (compare with **Figure 4B**), suggesting that the main promoter activity of Pfl01_2810-*tspo* lies in this region. By contrast, no activity was detected at each temperature for the two other constructs during the exponential growth phase. To get further insight into the role of RpoH in Pfl01_2810 – *tspo* transcription, the pHK-TSPO-157 reporter fusion and the pAB133 empty vector were transferred into *P. aeruginosa* PAO1 and in its *rpoH* isogenic mutant strain (Jacobs et al., 2003). *P. aeruginosa* wild type and *rpoH* mutant growth at 37°C was similar. As in the case of *P. fluorescens* Pf0-1, the promoter region activity increased during the growth phase of wild-type *P. aeruginosa* and then decreased. As shown on **Figure 6**, the activity of this promoter region was reduced by about 25% in *P. aeruginosa* *rpoH* mutant strain, suggesting that RpoH may be at least partly involved in this phenotype.

Discussion

Based on the Pseudomonas database (Winsor et al., 2011), *P. fluorescens* Pfl01_2810 – *tspo* was predicted to form an operonic structure with the Pfl01_2810 gene encoding a putative hybrid histidine kinase. We confirm in this study that the two genes are indeed co-transcribed, suggesting that both genes are genetically, but also possibly functionally linked. This latter hypothesis is supported by a previous STRING functional analysis (Leneveu-Jenvrin et al., 2014). Histidine kinases are often associated with their response regulator, which contains a receiver or response regulator domain, forming a two component signal transduction system. Hybrid-type histidine kinases comprise a histidine kinase with a receiver domain within one molecule (Stock et al., 2000). It is conceivable that TSPO could participate in the signaling-transduction pathway triggered by Pfl01_2810. Such a functional link between TSPO and a histidine kinase has been previously suggested in other bacteria. For example, in the symbiotic rhizosphere bacterium *Sinorhizobium meliloti*, TSPO has been shown to interact with the sensor FixL of an oxygen-sensing two-component system, regulating nutrient deprivation-induced genes under low oxygen

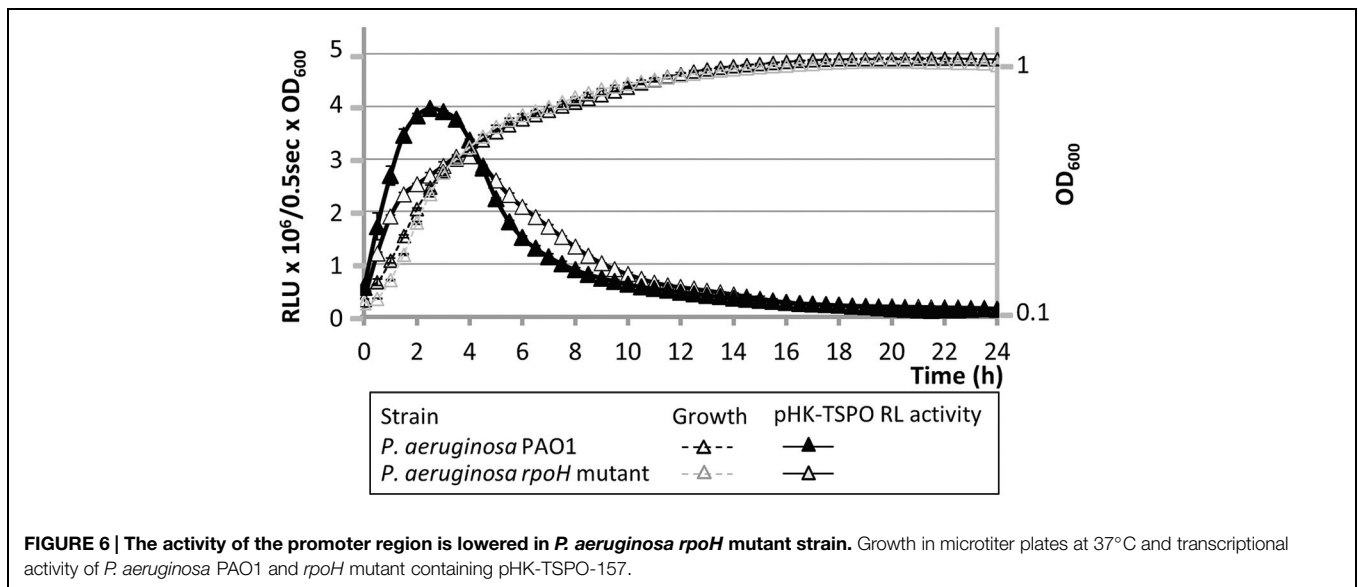


FIGURE 6 | The activity of the promoter region is lowered in *P. aeruginosa rpoH* mutant strain. Growth in microtiter plates at 37°C and transcriptional activity of *P. aeruginosa* PAO1 and *rpoH* mutant containing pHK-TSPO-157.

tension (Davey and de Bruijn, 2000). In the photosynthetic bacterium *Rhodobacter sphaeroides*, TSPO has been shown to regulate the expression of bacteriochlorophyll biosynthetic genes by controlling levels of tetrapyrroles in response to the simultaneous presence of light and oxygen (Yeliseev et al., 1997; Yeliseev and Kaplan, 1999, 1995). Little data are actually available about the function of Pfl01_2810 putative histidine kinase. In fact, no Pfl01_2810 homolog gene was predicted in the sequenced genomes of *Pseudomonas fluorescens* strains (<http://www.pseudomonas.com>, Winsor et al., 2011), suggesting that the association between Pfl01_2810 and *tspo* was specific to *P. fluorescens* Pf0-1. A Pfl01_2810 homolog was, however, predicted in 22 sequenced genomes of *Pseudomonas* species, among which *P. putida* (nine strains), *P. syringae* (six strains), *P. stutzeri* (five strains), *P. entomophila* (one strain), and *P. poae* (one strain). In these strains, the Pfl01_2810 homolog gene is often clustered with a (GntR-, LysR- or TetR-like) transcriptional regulator, and with a cytochrome *c* oxydase or an iron-sulfur cluster binding protein encoding gene in case of *P. putida* strains, or a cation transporter encoding gene in case of *P. stutzeri* and some *P. syringae* strains (Winsor et al., 2011). Taken together, these data may suggest a link between Pfl01_2810 and the oxidative level homeostasis, that should be investigated in future studies.

A transcriptional fusion approach was used herein to get further insights into *tspo* expression in *P. fluorescens* Pf0-1. We show that Pfl01_2810 – *tspo* cluster transcription was growth-phase dependent and transient, since a peak was observed in the early exponential phase when bacteria were grown in LB medium at 28°C. These data raised the possibility that TSPO may only be temporarily required by the cells during the exponential growth phase and hence possibly rapidly down-regulated after induction, suggesting that expression of these two genes was tightly regulated. Accordingly, TSPO was shown to be transiently induced in the *Arabidopsis* plant cell where it is one of the most strongly induced “early” genes (Kreps et al., 2002; Seki et al., 2002;

Zimmermann et al., 2004; Winter et al., 2007; Guillaumot et al., 2009a; Hermans et al., 2010; Vanhee and Batoko, 2011; Vanhee et al., 2011). Similar transient expression of *tspo* has been reported in *Physcomitrella patens* following chitosan treatment leading to the generation of an oxidative stress. In this moss plant, *tspo* expression was indeed induced by four- to fivefold about 15 to 30 min following treatment, and then decreased rapidly about 90 min after treatment (Lehtonen et al., 2012).

In plants, AtTSPO has been suggested to participate to the global plant water stress response, since its level increases in plants producing ABA, the phyto-hormone that regulates plant water status through regulation of stomatal closure (Finkelstein et al., 2002; Nambara and Marion-Poll, 2005), *tspo* being one of the ABA-responsive genes (Guillaumot et al., 2009a,b; Balsemão-Pires et al., 2011). Here we demonstrate that growth of *P. fluorescens* Pf0-1 in high salinity and osmolarity conditions leads to an increase in the operonic structure transcription, at least partly through activity of AlgU. AlgU is the major ECF sigma factor that is involved in maintaining the cell wall integrity and triggers the global response to cell wall damages in *Pseudomonas* species (Wood and Ohman, 2009). For example, it has been shown that in *P. protegens* CHAO, AlgU favors bacterial resistance to desiccation and osmotic stress (Schnider-Keel et al., 2001). Taken together, our results suggest that Pfl01_2810-*tspo* operon is part of the cell wall stress response triggered by AlgU. Through a bioinformatic study using MEME algorithm, we could, however, not detect an AlgU binding motif, suggesting that the effect of AlgU on Pfl01_2810 – *tspo* expression could be indirect. However, a sequence that presents a high level of similarity with the binding site for the alternative sigma factor RpoH was detected in this region, suggesting that RpoH could be involved directly in the operonic structure transcription. This hypothesis is supported by (i) the increased transcription of the operonic structure when bacteria are grown at high temperature, (ii) the increased activity of the reporter fusion containing the RpoH putative binding motif, but not of the other ones in

which this region was absent, (iii) the reduced promoter activity in a *P. aeruginosa* *rpoH* mutant strain, and finally, (iv), the fact that RpoH itself is part of AlgU regulon, since at least one of its identified promoters was AlgU-dependent (Schurr et al., 1996; Keith and Bender, 1999). However, the promoter activity was reduced by only 25% in *P. aeruginosa* *rpoH* mutant, suggesting that RpoH was not the main actor in the promoter activity, at least in *P. aeruginosa*. *In vitro* electrophoretic mobility gel-shift assay or yeast-one hybrid assay should now be used to confirm the binding of RpoH to its predicted promoter site. Taken together, these data suggest that Pfl01_2810-*tspo* is mainly regulated by AlgU, and to a lower extent by RpoH, through a direct or indirect molecular mechanism. Interestingly, two alternative sigma factors RpoH(I) and RpoH(II) were identified in *R. sphaeroides*, RpoH(I) being mainly involved in the reactive singlet oxygen response, and RpoH(II) in the heat stress response. Through a global predictive study, Nuss et al. (2010) identified a conserved binding sequence for RpoH(II) upstream *R. sphaeroides* *tspo*. Interestingly, in this bacterium, RpoH(II) is controlled by RpoE, the AlgU homolog ECF sigma factor in *R. sphaeroides* (Nuss et al., 2010; Dufour et al., 2012). The link between RpoH and *tspo* is further strengthened by the global transcriptomic study performed on the symbiotic bacterium *Synorhizobium meliloti* during heat shock and stationary phase growth (Barnett et al., 2012). These bacteria possess two RpoH-like sigma factors, i.e., RpoHI that is the major player in the heat shock response, and RpoHIII that is mainly involved during the stationary growth phase. Interestingly, the *tspo* gene has been reported to be induced in a RpoHI-dependent manner (Supplementary Table S1 in Barnett et al., 2012). Taken together, our data support that the operonic structure Pfl01_2810 – *tspo* belongs to the AlgU/RpoH regulons.

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Author Contributions

Conceived and designed experiments: EB, NC, CL-J, SC. Performed the experiments: CL-J, EB, OM. Analyzed the data: CL-J, EB, NC, SC. Wrote the paper: CL-J, PC, NC, MF, SC. All authors read and approved the final manuscript.

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