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Agnès SKAPSKI

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Exploration fonctionnelle du génome de Mycoplasma agalactiae : Recherche des facteurs de virulence en culture cellulaire

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JURY

M. Matthieu ARLAT (Président)	Professeur à l'Université Paul Sabatier de Toulouse
Mme Caroline LEROUX (Rapporteur)	Directeur de Recherche à l'INRA de Lyon
M. Joachim FREY (Rapporteur)	Professeur à l'Université de Berne, Suisse
Mme Cécile BEBEAR	Professeur à l'Université Victor Ségalen de Bordeaux
Mme Christine CITTI	Directeur de Recherche à l'INRA de Toulouse
M. Eric BARANOWSKI	Chargé de Recherche à l'INRA de Toulouse

Ecole doctorale : Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries (SEVAB)

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Directeur(s) de Thèse : Mme Christine CITTI et M. Eric BARANOWSKI

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LISTE DES ABREVIATIONS

ARNnc : ARN non codant

ARNm : ARN messager

CARDS TX : Community-Acquired Respiratory Distress Syndrome Toxin

CHP : Conserved Hypothetical Protein

Drp : DUF285 Related Protein

DUF : Domain of Unknown Function

HGT : Horizontal Gene Transfer

HP : Hypothetical Protein

ICE : Integrative Conjugative Element

NCR : Non Coding Region

STM : Signature Tag Mutagenesis

TIGEF : T Immortalized Goat Embryo Fibroblast

TIGMEC : T Immortalized Goat Milk Epithelial Cell

Tn : Transposon

Vpma : Variable Protein of *Mycoplasma agalactiae*

Introduction

INTRODUCTION

Les mycoplasmes sont des bactéries atypiques qui suscitent un intérêt croissant au sein de la communauté scientifique. Isolés pour la première fois en 1898 (Nocard et Roux, 1898), l'étude de ces organismes a été freinée par des contraintes liées à leur culture en laboratoire (Razin et Hayflick, 2010). Des progrès récents ont permis de révéler l'importance de ces bactéries dont plusieurs espèces sont pathogènes pour l'homme ainsi que pour de nombreuses espèces animales et végétales (Razin et Hermann, 2002). Considérés comme des formes de vie minimales, les mycoplasmes soulèvent des questions importantes relatives aux mécanismes de survie et d'adaptation à l'hôte, de même qu'ils offrent une plateforme unique pour l'étude des bases du vivant (Gibson *et al.*, 2010).

1. Taxonomie et caractéristiques des mycoplasmes

La classe des *Mollicutes* regroupe l'ensemble des bactéries communément appelées mycoplasmes (Edward *et al.*, 1967). Divisée en 4 ordres et 5 familles (Tableau 1), elle compte actuellement plus de 200 espèces, dont une majorité appartient au genre *Mycoplasma* (Johansson et Pettersson, 2002; Euzéby, 2011).

Les mycoplasmes dérivent d'ancêtres communs aux bactéries à Gram positif et faible pourcentage en G+C (Razin *et al.*, 1998). Leur évolution est marquée par une réduction massive de leur génome, dont la taille varie entre 580 kpb et 2200 kpb, et par une perte importante d'information génétique. Cette évolution dite « régressive » est à l'origine du caractère atypique de ces organismes. Ce sont des bactéries de petite taille (0,15 à 0,45 µm), dépourvues de paroi suite à la perte des gènes intervenant dans la synthèse du peptidoglycane. A l'exception des organismes des groupes *Phytoplasma* et *Acholeplasma*, leur usage de certains codons diffère du code génétique universel : le codon UGA, qui sert de codon stop chez les bactéries « classiques », code pour le tryptophane chez les mycoplasmes. Enfin, ces organismes possèdent des capacités métaboliques limitées (Razin *et al.*, 1998). L'absence de voies métaboliques essentielles comme celles du cholestérol, sauf chez les Acholeplasmes, ou des acides aminés explique leur association étroite avec des hôtes plus complexes.

L'analyse des ARNs 16S de plusieurs espèces de *Mollicutes* a permis d'établir les relations phylogénétiques existant entre ces organismes (Fig. 1). Les *Mollicutes* ont été classés en cinq groupes. Les groupes *Hominis* et *Pneumoniae* rassemblent essentiellement des espèces isolées chez l'homme et un large spectre d'animaux alors que les groupes *Phytoplasma* et

Acholeplasma se composent d'espèces qui colonisent principalement les végétaux et leurs insectes vecteurs. Enfin, le groupe *Spiroplasma* réunit à la fois des espèces associées aux végétaux ou aux animaux, une partie de ces dernières formants le sous-groupe mycoides qui compte cinq mycoplasmes pathogènes pour les ruminants.

Tableau 1. Classification taxonomique des *Mollicutes* [a]

Ordre	Famille	Genre	Nombre d'espèces	Hôtes principaux
Mycoplasmatales	*Mycoplasmataceae*	*Mycoplasma* [b]	125	Animaux, Homme
		Ureaplasma	7	Animaux, Homme
Entomoplasmatales	*Entomoplasmataceae*	*Entomoplasma*	6	Insectes, Plantes
		Mesoplasma	12	Insectes, Plantes
	Spiroplasmataceae	*Spiroplasma*	38	Insectes, Plantes
Acholeplasmatales	*Acholeplasmataceae*	*Acholeplasma*	18	Animaux, Plantes
		Phytoplasma [c]	6	Insectes, Plantes
Anaeroplasmatales	*Anaeroplasmataceae*	*Anaeroplasma*	4	Animaux
		Asteroleplasma	1	Animaux

[a] D'après Euzéby, 2011 ; Johansson et Pettersson, 2002.
[b] Comprend les espèces hémotropes précédemment classées dans les genres *Eperythrozoon* et *Haemobartonella*.
[c] Genre composé d'espèces non cultivables ; phylogénétiquement proches des Acholeplasmes et provisoirement classées dans la famille des *Acholeplasmataceae*.

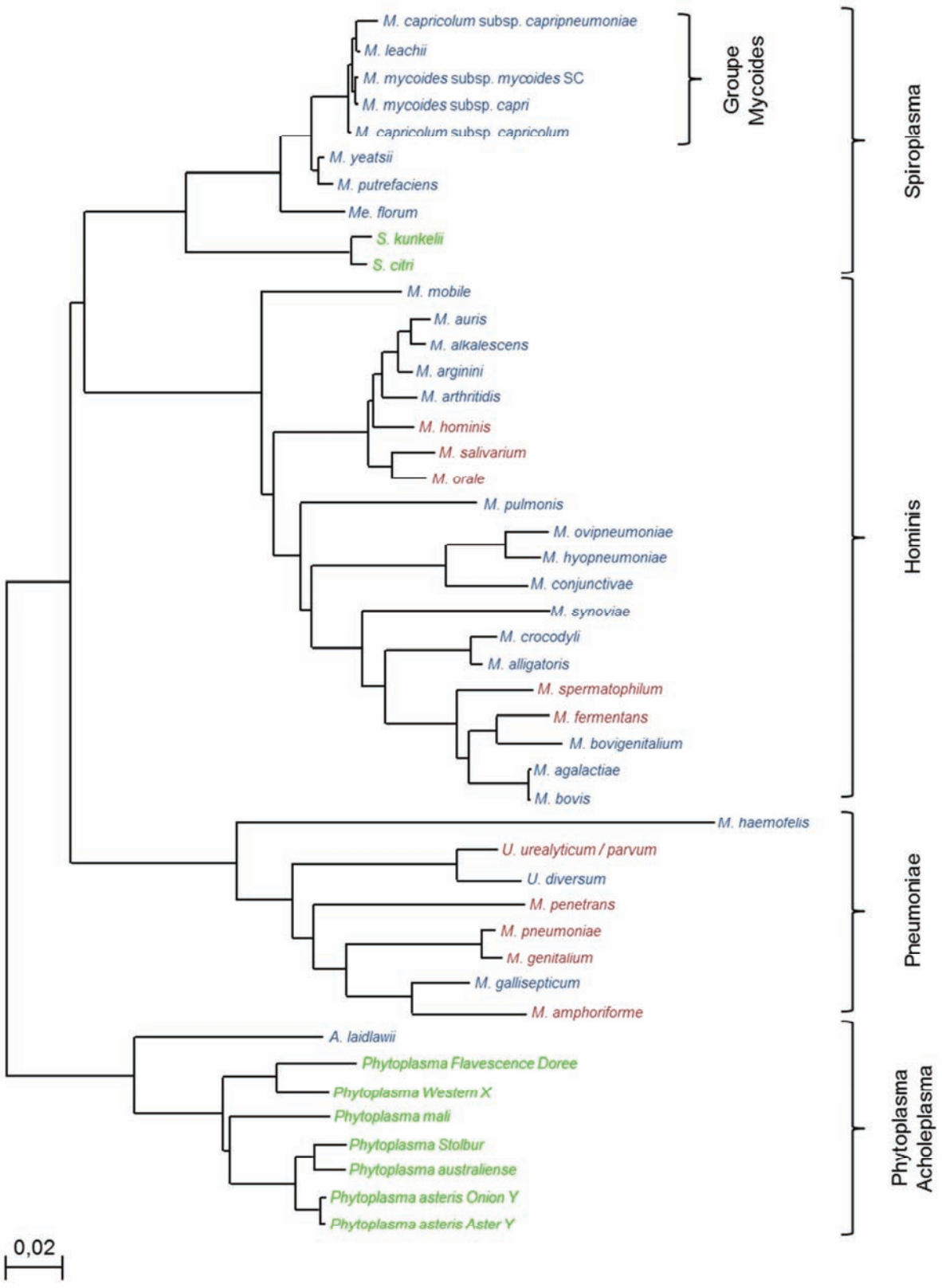

Figure 1. Arbre phylogénétique des *Mollicutes* basé sur l'analyse des séquences des ARNs 16S. L'échelle représente le pourcentage de substitutions nucléotidiques. Espèces colonisant les végétaux (vert) ; les animaux (bleu) ; l'homme (rouge). Adapté de : http://cbib1.cbib.u-bordeaux2.fr/molligen3b/SPECIES/phylo.php.

2. Pouvoir pathogène des mycoplasmes

Les microbiologistes ont longtemps douté du pouvoir pathogène des mycoplasmes, principalement en raison des difficultés techniques liées à leur isolement et leur identification. L'amélioration récente des techniques de diagnostic a permis de prendre conscience de l'importance sanitaire et économique de ces bactéries.

Mycoplasmoses en santé publique et en médecine vétérinaire

En santé humaine, les espèces pathogènes sont *M. pneumoniae* et *M. genitalium* qui colonisent principalement les voies respiratoires et uro-génitales (Blanchard et Bébéar, 2002). Des pathogènes opportunistes comme *M. penetrans* et *M. fermentans* ont été isolés chez des patients infectés par le virus de l'immunodéficience humaine (VIH) (Lo *et al.*, 1992; Shimizu *et al.*, 2004). Il existe également des espèces non pathogènes comme *M. salivarium* et *M. orale* (Blanchard et Bébéar, 2002).

Les mycoplasmoses animales sont généralement des maladies à faible mortalité, mais à forte morbidité (Baseman et Tully, 1997; Rosengarten *et al.*, 2001; Frey, 2002). Leur impact économique affecte l'ensemble des filières de production animale. Plus de 25 espèces de mycoplasmes ont été identifiées chez les ruminants (Tableau 2). La péripneumonie contagieuse bovine (PPCB), dont l'agent étiologique est *M. mycoides* subsp. *mycoides* Small Colony (SC), affecte les grands ruminants. Maladie à déclaration obligatoire inscrite sur la liste des maladies de l'Office International des Epizooties (OIE), la PPCB provoque de graves pneumopathies. Elle est endémique en Afrique et sévit de manière sporadique en Asie. En Europe, elle a été éradiquée au début du XX^{ème} siècle, mais de nombreux foyers ont ré-émergés en France et dans le sud de l'Europe dans les années 80. Les derniers foyers datent de 1999 au Portugal (Le Grand *et al.*, 2008). La vaccination à l'aide d'une souche vivante atténuée est utilisée en Afrique (Thiaucourt *et al.*, 2000). Un autre mycoplasme responsable d'importantes pertes économiques chez les grands ruminants est *M. bovis*, isolé dans la plupart des pays du monde (Le Grand *et al.*, 2008). Bien que ne faisant pas partie de la liste de l'OIE, *M. bovis* a une importance économique majeure car il est à l'origine de nombreuses pathologies dont des mammites, particulièrement aux Etats-Unis, et des bronchopneumonies infectieuses chez les veaux et les jeunes bovins (Pfützner et Sachse, 1996). A l'heure actuelle, aucun vaccin n'est disponible en Europe mais des vaccins commerciaux existent aux Etats-Unis bien que leur efficacité soit discutée (Nicholas *et al.*, 2009; Soehnlen *et al.*, 2011). Chez les petits ruminants, la pleuropneumonie contagieuse caprine (PPCC), causée par *M. capricolum* subsp. *capripneumoniae*, atteint les caprins. Inscrite sur la liste de l'OIE, la PPCC sévit en Afrique, au Moyen-Orient et en Asie (Thiaucourt et Bölske, 1996). Un vaccin inactivé

existe et permet la protection des animaux pendant environ un an (Thiaucourt *et al.*, 1996). Enfin, l'agalactie contagieuse des petits ruminants est une autre mycoplasmose importante inscrite sur la liste de l'OIE. Elle fera l'objet du paragraphe 3 de cette Introduction.

Les filières porcines et avicoles ne sont pas épargnées. *M. hyopneumoniae* est l'agent étiologique de la pneumonie enzootique porcine et le principal pathogène du complexe respiratoire porcin (Kobisch et Marois, 2008). Des vaccins adjuvés sont utilisés en France. Ils permettent de réduire les symptômes et les lésions pulmonaires. Les espèces *M. hyorhinis* et *M. hyosynoviae* provoquent des arthrites, mais aucun vaccin n'est disponible (Kobisch et Marois, 2008). La filière avicole est atteinte par des infections à *M. gallisepticum* et *M. synoviae*, qui figurent sur la liste de l'OIE, et à *M. meleagridis* et *M. iowae* (Stipkovits et Kempf, 1996). Ces quatre espèces sont à l'origine d'infections respiratoires, génitales ou articulaires. L'utilisation de vaccins inactivés ou vivants atténués permet de réduire les signes cliniques (Gautier-Bouchardon et Kempf, 2008).

L'existence de mycoplasmes pathogènes a également été mise en évidence chez les animaux domestiques. Chez le chien, *M. cynos* est associé à des pneumonies, *M. haemocanis* (auparavant *Haemobartonella canis*) à des anémies et *M. canis* à des infections uro-génitales (Chalker, 2005). Chez le chat, *M. felis* provoque des conjonctivites et des infections respiratoires ; *M. haemofelis* (auparavant *Haemobartonella felis*) est à l'origine d'anémies (Campbell *et al.*, 1973; Haesebrouck *et al.*, 1991; Tasker, 2010).

Outre l'existence d'espèces de mycoplasmes pathogènes, un certain nombre d'espèces non pathogènes (*M. bovirhinis*), opportunistes ou peu pathogènes (*M. arginini*, *M. ovis*) ont été identifiées. Pour d'autres, le pouvoir pathogène demeure incertain (*M. canadense*, *M. alkalescens*). Enfin certaines espèces ont fait preuve d'un faible pouvoir pathogène lors d'infections expérimentales sans que leur impact ne soit clairement défini sur le terrain (*M. dispar*, *M. conjunctivae*, *M. ovis*).

Risque zoonotique

Il est généralement admis que les mycoplasmes ont un spectre d'hôte étroit (Razin *et al.*, 1998) mais la détection de *M. canis*, pathogène du chien, chez des ruminants (ter Laak *et al.*, 1993) ou de *M. felis*, pathogène du chat, chez des chevaux (Wood *et al.*, 1997) souligne la possibilité de transmission entre animaux domestiques et animaux de rente et pose la question de la transmission à l'homme. Quelques cas de contaminations par des mycoplasmes animaux ont été rapportés chez l'homme mais sans que l'hypothèse d'une zoonose ne puisse être suspectée (Pitcher et Nicholas, 2005).

Tableau 2. Principaux mycoplasmes de ruminants [a]

Espèces	Virulence	Hôte [b]	Tropisme	Symptômes associés [c]
M. mycoides subsp. mycoides SC	Très pathogène	Bovins	Poumons	Pleuropneumonie
M. capricolum subsp. capripneumoniae	Très pathogène	Caprins	Poumons	**Pleuropneumonie**
M. capricolum subsp. capricolum	Très pathogène à pathogène	Caprins > ovins >> bovins	Mamelle Articulation Poumon Oreille [e]	Mammite **Arthrite** **Pneumonie** (Kérato-conjonctivite)
M. mycoides subsp. capri / M. mycoides subsp. mycoides LC [d]	Très pathogène à pathogène	Caprins > ovins >> bovins	Mamelle Poumon Articulation Œil Oreille [e]	**Mammite** **Pneumonie** **Arthrite** (Kérato-conjonctivite)
M. bovis	Pathogène	Bovins	Mamelle Articulation Poumon Appareil génital Oreille interne Œil	**Mammite** **Arthrite** **Pneumonie** (Avortement) (Otite) (Kérato-conjonctivite)
M. leachii	Pathogène	Bovins	Mamelle Articulation Appareil génital	**Mammite** (Arthrite) (Avortement)
M. agalactiae	Pathogène à peu pathogène	Ovins Caprins	Mamelle Articulation Poumon Œil Appareil génital Oreille [e]	**Mammite** Arthrite (Pneumonie) Kérato-conjonctivite
M. californicum	Peu pathogène	Bovins	Mamelle Articulation	(Mammite) Arthrite
M. conjunctivae	Peu pathogène	Ovins	Œil	**Kérato-conjonctivite**

(Suite page suivante)

Tableau 2 (suite)

Espèces	Virulence	Hôte [b]	Tropisme	Symptômes associés [c]
M. bovigenitalium	Peu pathogène	Bovins	Poumon Mamelle Appareil génital	(Mammite) (Infertilité)
M. putrefaciens	Peu pathogène	Caprins > ovins	Mamelle Articulation Poumon Œil Oreille [e]	**Mammite** **Arthrite** Pneumonie (Kérato-conjonctivite)
M. ovipneumoniae	Peu pathogène	Ovins	Poumon	**Pneumonie**
M. canadense	Pouvoir pathogène mal connu ou seulement certaines souches pathogène	Bovins	Mamelle Articulation Poumon	(Mammite)
M. alkalescens	Pouvoir pathogène mal connu ou seulement certaines souches pathogène	Bovins	Mamelle Articulation Poumon	(Mammite) (Arthrite)
M. dispar	Pouvoir pathogène mal connu ou seulement certaines souches pathogène	Bovins	Poumons	(Bronchopneumonie)
M. wenyonii	Pouvoir pathogène mal connu ou seulement certaines souches pathogène	Bovins	Sang	Anémie
M. ovis	Peu pathogène à non pathogène	Ovins	Sang	**Anémie**
M. bovirhinis	Non pathogène	Bovins	Poumon	-
M. arginini	Non pathogène	Bovins Ovins Caprins	Poumon Mamelle Appareil génital	-

[a] D'après (Citti, 2006).
[b] Les symboles > et >> indiquent les hôtes infectés majoritairement ou très majoritairement.
[c] Les symptômes majoritairement rencontrés sont indiqués en caractères gras; les symptômes souvent rencontrés sont indiqués en police normale ; les symptômes rares sont indiqués entre parenthèses.
[d] *M. mycoides* subsp. *mycoides* LC est aujourd'hui regroupé au sein du taxon *M. mycoides* subsp. *capri* (Manso-Silván et *al.*, 2009)
[e] Un portage auriculaire a été identifié.

3. Agalactie contagieuse des petits ruminants

L'agalactie contagieuse est une maladie des petits ruminants inscrite sur la liste de l'OIE. Elle est responsable de pertes économiques conséquentes, particulièrement dans les pays où les ovins et les caprins produisent une part importante des ressources en lait et en viande (Frey, 2002). Maladie répandue dans le monde entier, cette mycoplasmose est la plus fréquente chez les petits ruminants en Europe et sur le pourtour du bassin méditerranéen (Bergonier *et al.*, 1997). Le Royaume-Uni et l'Australie demeurent indemnes malgré une forte concentration de petits ruminants.

Isolé pour la première fois en 1923 (Bridré et Donatien, 1923), l'agent étiologique historique de l'agalactie contagieuse est *M. agalactiae* qui fait partie du groupe phylogénétique *Hominis* (Fig. 1) (Bergonier *et al.*, 1997). Ce mycoplasme est principalement isolé chez les ovins, mais des isolements chez des caprins domestiques ou sauvages ont été rapportés (Bergonier *et al.*, 1997; González-Candela *et al.*, 2006; Verbisck-Bucker *et al.*, 2008).

L'agalactie contagieuse des petits ruminants peut également être provoquée par d'autres espèces de mycoplasmes du groupe phylogénétique *Spiroplasma* : *M. mycoides* subsp. *mycoides* Large Colony (LC) qui est aujourd'hui regroupé au sein du taxon *M. mycoides* subsp. *capri* (Manso-Silván *et al.*, 2009), *M. capricolum* subsp. *capricolum* et *M. putrefaciens*. Ces trois espèces, qui appartiennent au sous-groupe phylogénétique mycoides élargi (Fig. 1), sont principalement isolées chez les caprins.

Aspects cliniques et transmission

Le syndrome de l'agalactie contagieuse provoqué par *M. agalactiae* est caractérisé par des mammites, des arthrites et des kérato-conjonctivites qui apparaissent la plupart du temps de manière dissociée (Fig. 2) (Bergonier *et al.*, 1997; Citti, 2006). *M. mycoides* subsp. *mycoides* LC, *M. capricolum* subsp. *capricolum* et *M. putrefaciens* sont à l'origine de signes cliniques très similaires (Thiaucourt, 2003). Des signes pulmonaires, plus rares, sont parfois mis en évidence chez les jeunes animaux ainsi que des épisodes de vulvovaginites granuleuses chez les chèvres (Singh *et al.*, 1974; Bergonier et Poumarat, 1997). L'existence d'un portage auriculaire, généralement asymptomatique, est également connue (Bergonier *et al.*, 1997).

La transmission de la maladie se fait essentiellement de façon directe et horizontale. L'excrétion de mycoplasmes pathogènes peut avoir lieu dans le lait, les sécrétions respiratoires ou uro-génitales, les larmes ou les fèces. La question d'une transmission indirecte, via des insectes vecteurs a également été soulevée (Bergonier *et al.*, 1997).

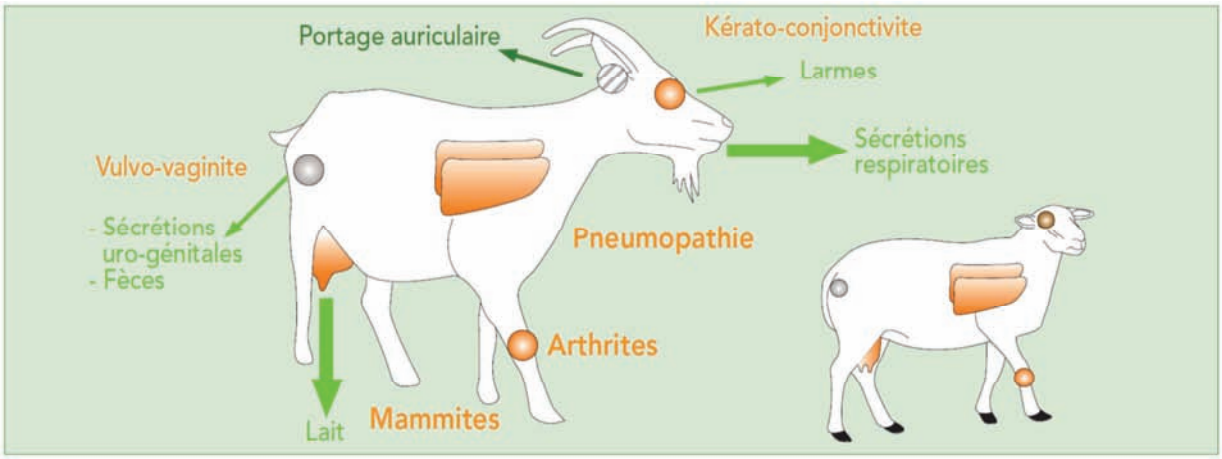

Figure 2. Symptômes de l'agalactie contagieuse chez les petits ruminants. Les symptômes sont indiqués en orange et les voies d'excrétion en vert. D'après Citti, 2006.

Figure n°2 reprise avec l'aimable autorisation de N ÉVA- NOUVELLES ÉDITIONS VÉTÉRINAIRES ET ALIMENTAIRES.

Traitement et prophylaxie

Le traitement de l'agalactie contagieuse repose sur l'utilisation d'antibiotiques actifs contre les bactéries dépourvues de paroi. Les principales molécules utilisées sont les tétracyclines, les phénicolés, les fluoroquinolones et les macrolides (Bergonier et Poumarat, 1997). L'objectif du traitement antibiotique est l'amélioration de l'état clinique des animaux car la guérison bactériologique est rarement atteinte.

Les vaccins disponibles sont pour la plupart des vaccins inactivés par la chaleur ou le formol et accompagnés ou non d'adjuvants immunogènes. Quels que soient la souche ou l'adjuvant utilisés, l'immunité conférée est partielle et implique des rappels rapprochés (Bergonier et Poumarat, 1997). Ces vaccins ne protègent pas contre l'infection et aucun n'est autorisé sur le marché français. L'Agence Française de Sécurité Sanitaire des Aliments (AFSSA) a été saisie en 2010 d'une demande d'avis relatif à la vaccination contre l'agalactie contagieuse afin de statuer sur l'efficacité des vaccins commercialisés en Espagne, en vue d'une éventuelle commercialisation sur le territoire français (Saisine n° 2010-SA-0105). En France, une prophylaxie sanitaire a été mise en place. Elle est basée sur le suivi des troupeaux et la déclaration obligatoire des cas suspectés avec confirmation au laboratoire ainsi que sur l'isolement ou l'abattage total des troupeaux infectés. Ces mesures ont permis l'assainissement de la Savoie et de la Haute Savoie qui sont considérées comme indemnes depuis 2002, mais la persistance de la maladie dans les Pyrénées Atlantiques met en évidence les limites de ce plan de contrôle.

Reproduction expérimentale de l'agalactie contagieuse

Différentes études de reproduction expérimentale de l'agalactie contagieuse ont été publiées (Tableau 3). Elles reposent sur l'infection de l'hôte naturel par *M. agalactiae*, en utilisant différentes voies d'inoculation. Le suivi de l'infection est basé sur l'étude des signes cliniques, du niveau d'excrétion dans le lait, du niveau d'infection des nœuds lymphatiques ou de la réponse en anticorps. Lors de ces études, les doses infectieuses utilisées sont généralement élevées, de l'ordre de 10^7 à 10^9 unités formant colonies (UFC). Des brebis lactantes inoculées par voie intra-mammaire ont développé des mammites (Sanchis *et al.*, 2000). Les autres voies d'inoculation utilisées ont provoqué peu, voire pas de signes cliniques. L'infection par *M. agalactiae* induit généralement une réponse en anticorps et la détection du mycoplasme dans les nœuds lymphatiques proches du site d'inoculation. Toutefois, des problèmes de reproductibilité existent, les animaux infectés en parallèle ne développant pas toujours des signes cliniques ou des niveaux d'infection identiques. Ces travaux ont néanmoins abouti à la mise au point de différents modèles permettant l'étude de l'agalactie contagieuse ainsi que la comparaison de différentes souches de *M. agalactiae*.

Tableau 3. Modèles de reproduction expérimentale de l'agalactie contagieuse

Modèle	Voies d'inoculation	Références
Chèvre	Intra-mammaire	(Hasso *et al.*, 1993)
	Intra-veineuse	(Hasso *et al.*, 1993)
	Sous-cutanée	(Hasso *et al.*, 1993)
	Orale	(Hasso *et al.*, 1993)
Brebis en lactation	Intra-mammaire	(Sanchis *et al.*, 2000)
	Sous-cutanée	D. Bergonier, communication personnelle
Agneaux	Intraveineuse	(MacOwan *et al.*, 1984)
	Sous-cutanée	(MacOwan *et al.*, 1984)
	Intra-nasale	(MacOwan *et al.*, 1984)
	Intra-trachéale	(MacOwan *et al.*, 1984)
	Conjonctivale	(Sanchis *et al.*, 1998)
Moutons	Intra-nasale	(Buonavoglia *et al.*, 1999)
	Conjonctivale	(Buonavoglia *et al.*, 1999)

4. L'espèce *Mycoplasma agalactiae*

Caractéristiques moléculaires

La séquence complète du génome de la souche de référence PG2 a été obtenue en 2007 (Sirand-Pugnet *et al.*, 2007). Cette souche possède un génome typique de *Mollicutes*, avec une taille réduite, un faible pourcentage en G+C et une forte densité de gènes (Tableau 4). L'annotation du génome a permis d'identifier 751 CDSs parmi lesquelles 404 ont été annotées avec une fonction potentielle. Les CDSs restantes codent pour des protéines hypothétiques conservées (CHP) qui présentent des homologies avec des protéines dont la fonction est inconnue ou des protéines hypothétiques (HP) sans homologie apparente avec d'autres protéines. Une analyse de protéomique réalisée sur une culture de PG2 a permis de détecter l'expression de 357 CDSs (Nouvel *et al.*, 2010).

En l'absence de paroi, les protéines membranaires sont à l'interface directe entre le mycoplasme et l'environnement. Elles sont donc susceptibles de jouer un rôle majeur dans les interactions qui ont lieu entre le mycoplasme et son hôte. Les lipoprotéines, ancrées dans la membrane plasmique par un phospholipide, et les protéines transmembranaires, comme les transporteurs, constituent les principales protéines associées à la membrane des mycoplasmes. Dans le génome de PG2, la plupart des 66 lipoprotéines sont annotées comme hypothétiques. Les lipoprotéines du système Vpma (Glew *et al.*, 2000), codées par 6 gènes, sont impliquées dans la variabilité de surface de *M. agalactiae* (Citti *et al.*, 2010). Les lipoprotéines P30, P40, P48 et P80 sont des antigènes importants (Rosati *et al.*, 1999, 2000; Tola *et al.*, 2001; Fleury *et al.*, 2001, 2002).

Plusieurs CDSs présentent des similarités avec les systèmes de restriction/modification (R/M) (Sirand-Pugnet *et al.*, 2007). Un locus composé de 6 gènes, nommés *hsd*, présente des homologies avec les systèmes R/M de type I.

Enfin, la souche PG2 possède un locus composé de 20 CDSs, parmi lesquelles une majorité de pseudogènes. Ces CDSs présentent des similarités avec des gènes identifiés dans des éléments conjugatifs intégratifs (Integrative Conjugative Element, ICE) et constituent un vestige d'ICE (Marenda *et al.*, 2005; Sirand-Pugnet *et al.*, 2007; Nouvel *et al.*, 2010).

Transfert horizontal de gènes

Une analyse détaillée du génome de la souche PG2 a montré que 18 % du génome ont été échangé par transferts horizontal avec des espèces du groupe mycoides (Sirand-Pugnet *et al.*, 2007). L'acquisition de gènes par transfert horizontal est plutôt surprenante chez des organismes dont l'évolution est marquée par une réduction de leur génome. Ces transferts ont eu lieu entre des espèces qui, bien que phylogénétiquement éloignées, partagent le même

hôte animal (Sirand-Pugnet *et al.*, 2007). Environ 30 % des gènes échangés codent pour des protéines membranaires qui pourraient jouer un rôle important dans les interactions avec l'hôte. Les gènes échangés pourraient avoir favorisé l'adaptation du mycoplasme à son hôte (Sirand-Pugnet *et al.*, 2007)

Plasticité génétique et mobilome

La séquence complète du génome de la souche *M. agalactiae* 5632 a été obtenue en 2010 (Nouvel *et al.*, 2010). Le génome de cette souche possède un contenu en G+C et une densité de gènes similaires à ceux de la souche PG2, mais sa taille est plus importante (Tableau 4). Il compte 826 CDS parmi lesquelles 505 ont été annotées avec une fonction potentielle. Une analyse de protéomique a permis d'identifier l'expression de 453 CDSs en milieu axénique, dont 313 sont communes aux deux souches (PG2 et 5632).

Globalement, le génome de la souche 5632 présente une synténie presque parfaite avec le génome de PG2 sans réarrangement majeur, mais un certain nombre de régions sont tout de même extrêmement différentes. Elles représentent la plus grande partie des différences de taille observées entre les génomes des souches PG2 et de 5632. La souche 5632 présente ainsi 3 éléments intégratifs conjugatifs complets, nommés ICE_{5632}-I à III et 2 vestiges, nommés ICE_{5632}-IV et V (Nouvel *et al.*, 2010). La souche 5632 contient également de multiples copies de séquences d'insertion (IS) qui n'existent pas chez PG2 mais qui sont présentes dans les génomes de *M. bovis* et *M. mycoides* subsp. *mycoides* SC. Ces IS sont impliquées dans la plasticité et la dynamique des génomes. Quatre CDSs codant pour des systèmes de R/M de type II ont été identifiées dans le génome de 5632, en plus des CDSs codant pour le système de type I identifié chez PG2 (Nouvel *et al.*, 2010). Enfin, la souche 5632 possède 103 gènes codant pour des lipoprotéines (Nouvel *et al.*, 2010). Parmi celles-ci, les Vpmas sont codées par 23 gènes, contre 6 dans la souche PG2, répartis dans deux régions différentes du génome. Cette organisation permet une plus grande diversité de surface.

Le génome de *M. agalactiae* est donc très dynamique et une souche donnée n'est pas forcément représentative de l'espèce dans son ensemble.

Tableau 4. Principales caractéristiques moléculaires du génome de *M. agalactiae* [a]

Caractéristiques	Souches	
	PG2	5632
Taille du génome (kpb)	877	1006
% G+C	29,70	29,62
Densité de gènes (%)	88,0	88,7
Nombre de CDS	751	826
CDS avec une fonction prédite	404	505
Protéines hypothétiques conservées	136	150
Protéines hypothétiques	166	148
Numéro d'accession GenBank	CU179680	FP671138
Numéro d'accession Refseq	NC_009497	NC_013948

[a] D'après Sirand-Pugnet *et al.*, 2007 ; Nouvel *et al.*, 2010

5. Facteurs de virulence et bactéries minimales

Les facteurs moléculaires responsables de la virulence des mycoplasmes présentent, sur la base de l'analyse des génomes, peu ou pas de similitudes avec les facteurs déjà décrits chez d'autres bactéries. Les mycoplasmes semblent avoir développé des mécanismes spécifiques qui leur permettent de faire face aux défenses immunes de l'hôte.

Etude des facteurs de virulence chez les bactéries minimales

Les premières études comparatives menées sur des souches exprimant différents degrés de virulence ont permis d'identifier quelques-uns des mécanismes d'expression du pouvoir pathogène chez les mycoplasmes. Cette approche a notamment été utilisée sur les espèces *M. mycoides* subsp. *mycoides* SC, *M. bovis* ou *M. pneumoniae* (Meseguer *et al.*, 2003; Thomas *et al.*, 2003a, 2003b; Krunkosky *et al.*, 2007; Bischof *et al.*, 2008).

L'essor de la génomique a ouvert de nouvelles perspectives pour l'étude des facteurs de virulence. L'analyse *in silico* des premières séquences complètes de génomes de mycoplasmes a ainsi permis d'identifier quelques facteurs potentiellement impliqués dans la virulence de ces bactéries, comme le facteur CARDS TX identifié chez *M. pneumoniae* (Kannan *et al.*, 2005). Toutefois, l'analyse des génomes séquencés a rapidement montré les limites de ces études *in silico* chez les mycoplasmes.

La mutagénèse aléatoire et le criblage de banques de mutants sont actuellement les approches les plus utilisées pour étudier les facteurs de virulence bactériens. La mutagénèse physico-chimique a recours à l'irradiation aux ultraviolets ou à l'utilisation d'agents mutagènes. Ces techniques ont été utilisées chez les espèces *M. mobile* et *M. pneumoniae* (Hansen *et al.*, 1979; Miyata *et al.*, 2000). Toutefois, le défaut majeur de ces techniques est l'accumulation de mutations ponctuelles difficilement repérables dans le génome. Le développement de techniques de mutagenèse transpositionelle a permis de contourner ce problème. Les transposons Tn916 et Tn4001, ainsi que leurs dérivés (Halbedel et Stülke, 2007), ont été utilisés pour la construction de banques de mutants chez les espèces *M. genitalium*, *M. pneumoniae*, *M. gallisepticum*, *M. pulmonis* et *M. arthritidis* (Hutchison *et al.*, 1999; Whetzel *et al.*, 2003; Mudahi-Orenstein *et al.*, 2003; Winner *et al.*, 2003; Hudson *et al.*, 2006; Dybvig *et al.*, 2008; French *et al.*, 2008). La technique de « Signature-Tagged Mutagenesis » (STM), plus récente, permet d'identifier chaque mutant à l'aide d'une séquence spécifique d'ADN intégrée avec le transposon et de tester des groupes de mutants plutôt que des mutants individuels (Hensel *et al.*, 1995; Mazurkiewicz *et al.*, 2006).

La mutagenèse transpositionelle a largement été utilisée en microbiologie pour la recherche de gènes de virulence (Autret et Charbit, 2005; Mazurkiewicz *et al.*, 2006). Chez les

mycoplasmes, elle a surtout été employée pour l'étude des bases du vivant (Hutchison *et al.*, 1999; Glass *et al.*, 2006), mais a permis l'identification de gènes impliqués dans la mobilité, l'adhérence, ainsi que d'autres facteurs associés à la virulence, comme la déshydrogénase dihydrolipoamide chez *M. gallisepticum* (Mudahi-Orenstein *et al.*, 2003; Hasselbring *et al.*, 2006; Hudson *et al.*, 2006).

Quelle que soit la technique de mutagenèse utilisée, l'identification de facteurs de virulence repose sur l'utilisation d'un crible de sélection adéquat. L'animal de laboratoire et la culture cellulaire sont les environnements les plus utilisés (Autret et Charbit, 2005). Les modèles cellulaires, plus simples à mettre en œuvre, sont une alternative aux études *in vivo* mais les résultats obtenus doivent être confirmés chez l'hôte naturel. Les cellules primaires offrent un environnement proche de l'hôte. Cependant des problèmes de dégénérescence apparaissent souvent après un nombre limité de passages. Le recours à des lignées cellulaires immortalisées permet de contourner le phénomène de dégénérescence mais les cellules de ces lignées sont plus éloignées de l'hôte naturel dont elles conservent peu de caractéristiques.

Variabilité antigénique et variabilité de surface

Les mycoplasmes ne possèdent pas de paroi et leur membrane plasmique est en contact direct avec l'environnement. Les protéines membranaires assurent donc directement les échanges avec le milieu extérieur et sont la cible de la réponse immune de l'hôte. Afin d'assurer leur survie et d'échapper à la réponse humorale, les mycoplasmes ont développé une stratégie qui repose sur des variations à haute fréquence des protéines de surface ainsi que sur des modifications de leur taille et/ou de leur structure (Citti *et al.*, 2005, 2010). Différents systèmes sont mis en œuvre par les mycoplasmes pour assurer ces variations aléatoires d'expression des protéines.

Le premier mécanisme, décrit chez *M. hyorhinis*, repose sur l'insertion ou la délétion d'un ou de plusieurs nucléotides dans la région promotrice des gènes codants pour les protéines de surface Vlps (Citti et Wise, 1995; Yogev *et al.*, 1995). Ces variations affectent directement et de façon drastique la transcription du gène situé en aval du promoteur. Un système similaire a été identifié chez *M. gallisepticum* (Markham *et al.*, 1994; Glew *et al.*, 1998). Un second mécanisme a été identifié chez *M. agalactiae* (gènes *vpma*), *M. bovis* (gènes *vsp*) et *M. pulmonis* (gènes *vsa*). Une recombinase permet une inversion des brins d'ADN entre des sites de recombinaison spécifiques, positionnant de façon alternative des gènes silencieux derrière un promoteur actif (Bhugra *et al.*, 1995; Lysnyansky *et al.*, 1999; Shen *et al.*, 2000; Lysnyansky *et al.*, 2001; Glew *et al.*, 2002; Chopra-Dewasthaly *et al.*, 2008). Un système

identique, basé sur des réarrangements de la région promotrice devant les gènes *mlp*, a été mis en évidence chez *M. penetrans* (Röske *et al.*, 2001; Horino *et al.*, 2003). Enfin, *M. synoviae* possède un unique gène *vlhA* et de multiples pseudogènes répartis sur l'ensemble du génome (Noormohammadi *et al.*, 2000). Des recombinaisons entre ces pseudogènes et le gène *vlhA* permettent de remplacer une partie de la séquence du gène *vlhA* par la séquence des pseudogènes.

Des variations de taille et de structure des protéines exprimées sont également possibles. Ainsi chez *M. agalactiae*, des glissements de l'ADN polymérase sur les brins d'ADN provoquent des variations de taille des Vpmas, des recombinaisons homologues entrainent des variations de leur structure et des duplications de gènes permettent l'émergence de versions alléliques et de nouveaux produits (Glew *et al.*, 2002; Flitman-Tene *et al.*, 2003; Nouvel *et al.*, 2009; Citti *et al.*, 2010).

Les mécanismes décrits ici et les variations de taille ou de structure peuvent intervenir d'une façon simultanée, générant un répertoire très dynamique des protéines de surface (Citti *et al.*, 2010).

Adhésion aux surfaces épithéliales

L'adhésion des mycoplasmes aux surfaces mucosales est une étape importante pour l'infection et la colonisation de l'hôte (Razin *et al.*, 1998). Les espèces *M. pneumoniae* et *M. genitalium* possèdent une structure terminale « en pointe » où se concentrent les adhésines et autres protéines impliquées dans l'adhésion (Razin et Jacobs, 1992). Tous les mycoplasmes ne possèdent pas ce type de structure spécialisée mais sont néanmoins capables d'adhérer aux cellules de l'hôte. La lipoprotéine T (LppT) de *M. conjunctivae* est impliquée dans l'adhésion aux cellules synoviales ovines. Cette lipoprotéine présente un motif RGD (Arginine-Glycine-Aspartate) qui suggère la participation de certaines intégrines ovines dans l'adhésion du mycoplasme (Zimmermann *et al.*, 2010). Les récepteurs cellulaires utilisés par les mycoplasmes pour l'adhésion sont des macromolécules complexes de type sialoglycoconjugués et des glycolipides sulfatés (Razin et Jacobs, 1992; Zhang *et al.*, 1994).

Des études menées chez *M. gallisepticum*, *M. pneumoniae* et *M. genitalium* ont montré qu'une perte de l'adhésion était accompagnée d'une perte de la virulence (Baseman *et al.*, 1982; Krause *et al.*, 1982; Razin et Jacobs, 1992; Papazisi *et al.*, 2002).

Chez *M. agalactiae*, la lipoprotéine P40 (Fleury *et al.*, 2002) participe à l'adhésion mais n'est pas exprimée chez toutes les souches indiquant que d'autres protéines peuvent être impliquées dans ce processus. Les études menées chez *M. pneumoniae* ont montré que l'adhésion était un processus multifactoriel (Razin et Jacobs, 1992).

Invasion de la cellule hôte

Les mycoplasmes sont des parasites extracellulaires, mais des études récentes ont montré que certaines espèces possèdent la capacité de pénétrer dans les cellules non phagocytaires. L'invasion cellulaire a notamment été étudiée chez *M. penetrans* (Lo *et al.*, 1993; Andreev *et al.*, 1995). Elle a également été démontrée pour d'autres mycoplasmes comme *M. fermentans*, *M. genitalium*, *M. pneumoniae* et *M. gallisepticum* (Taylor-Robinson *et al.*, 1991; Baseman *et al.*, 1995; Winner *et al.*, 2000). L'invasion cellulaire est un processus complexe dont les acteurs restent encore à définir. L'intervention de la fibronectine a été suggérée pour l'internalisation de *M. penetrans* (Girón *et al.*, 1996) et de *M. pneumoniae* (Dallo *et al.*, 2002) par analogie avec des observations faites chez *Neisseria gonorrhoeae* (van Putten *et al.*, 1998) et *Staphylococcus aureus* (Dziewanowska *et al.*, 1999, 2000). La subversion du cytosquelette pourrait être une autre stratégie aboutissant à l'invasion des cellules de l'hôte (Winner *et al.*, 2000).

La capacité de *M. agalactiae* à envahir les cellules de l'hôte reste à démontrer.

Formation de biofilms

Dans leur environnement naturel, les bactéries peuvent proliférer de façon individualisée ou au sein d'une communauté enchâssée dans une matrice extracellulaire complexe principalement constituée de polymères polysaccharidiques et appelée biofilm (Costerton *et al.*, 1978, 1995, 1999; Branda *et al.*, 2005). *In vivo*, la formation du biofilm permet de résister au flux de la circulation sanguine et à la phagocytose. Le biofilm permet aux bactéries de mieux supporter les conditions de stress, comme la carence en nutriments et les variations de pH, et augmente la résistance aux antiseptiques et aux antibiotiques (Donlan et Costerton, 2002). Le biofilm aurait donc un rôle dans la persistance des bactéries dans l'environnement et chez l'hôte animal.

Certaines espèces de mycoplasmes (*M. bovis*, *M. putrefaciens*, *M. cottewii*, *M. yeastsii*, *M. mycoides* subsp. *mycoides* Small Colony et *M. pulmonis*) sont capables de former un biofilm sur une surface inerte (McAuliffe *et al.*, 2006). La formation de biofilm *in vivo* a été démontrée pour *M. pulmonis* (Simmons et Dybvig, 2009).

La souche NCTC 10123 de *M. agalactiae* est capable de former du biofilm *in vitro* (McAuliffe *et al.*, 2006).

Subversion de la réponse immune

Les mycoplasmes sont capables d'exercer des effets immunomodulateurs sur le système immunitaire de leur hôte.

En 1981, Geary *et al.* ont mis en évidence une toxine inflammatoire chez *M. bovis* qui serait capable d'activer le complément (Geary *et al.*, 1981). Des protéines membranaires de *M. fermentans* seraient également liées à l'activation du complément (Matsumoto *et al.*, 1998).

L'induction de la prolifération des lymphocytes B et T par *M. arthritidis* fait l'objet d'études détaillées (Cole *et al.*, 1996; Razin *et al.*, 1998). L'activation des lymphocytes T par une protéine présente dans du surnageant de culture a permis d'identifier un superantigène, nommé MAM pour « *Mycoplasma arthritidis* mitogen ». D'autres mycoplasmes, comme *M. pulmonis*, sont également capables d'activer les lymphocytes B et T (Razin *et al.*, 1998). Ce dernier induit aussi une augmentation de l'activité lytique des cellules NK (« Natural Killer ») (Lai *et al.*, 1994; Razin *et al.*, 1998). Les espèces *M. penetrans* et *M. fermentans* peuvent respectivement envahir et fusionner avec les lymphocytes T et induire la mort de ces cellules (Franzoso *et al.*, 1992; Lo *et al.*, 1993; Razin *et al.*, 1998).

De nombreuses espèces de mycoplasmes sont également capables d'interagir avec les monocytes et les macrophages. Cette interaction aboutit à la production de cytokines, comme l'interféron et les interleukines 2, 4 et 10 (Razin *et al.*, 1998).

Enfin, des études récentes ont montré que *M. gallisepticum* et *M. synoviae* possèdent une protéase capable de cliver les immunoglobulines de type G (Cizelj *et al.*, 2011).

Chez *M. agalactiae*, la protéine P48 (Rosati *et al.*, 1999, 2000) présente des motifs similaires à ceux de protéines membranaires de *M. fermentans* dont l'activité biologique est liée à l'activation du complément et à l'induction des cytokines (Kostyal *et al.*, 1994; Matsumoto *et al.*, 1998).

Production de toxines et de métabolites toxiques

Un facteur de virulence nommé CARDS TX pour « Community-Acquired Respiratory Distress Syndrome Toxin » a été identifié chez *M. penetrans* et *M. pneumoniae*. Cette toxine présente des similitudes avec des ADP-ribosyltransférases comme la toxine pertussique de *Bordetella pertussis* (Kannan *et al.*, 2005; Kannan et Baseman, 2006; Johnson *et al.*, 2009). Les ADP-ribosyltransférases induisent des modifications protéiques post-traductionnelles sur les protéines qui lient les nucléotides (Krueger et Barbieri, 1995). De telles modifications ont été observées en culture cellulaire avec une protéine CARDS TX recombinante (Kannan et

Baseman, 2006). Le facteur CARDS TX est à ce jour l'un des rares produits mycoplasmiques semblables à des toxines bactériennes plus classiques.

Des agents nécrotiques digérant les tissus ont été mis en évidence chez *M. alligatoris* (Brown *et al.*, 2004; Hunt et Brown, 2007). Il s'agirait d'une hyaluronidase et d'une sialidase, toutes deux présentant des homologies avec des protéines sécrétées par *Clostridium perfringens*. L'interaction entre hyaluronidase et sialidase serait nécessaire à l'expression du pouvoir pathogène chez *M. alligatoris*. *M. crocodyli* possède également une hyaluronidase mais pas de sialidase fonctionnelle ce qui pourrait expliquer son faible pouvoir pathogène en comparaison avec *M. alligatoris* (Brown *et al.*, 2004).

Enfin, la production de radicaux peroxydes ou superoxydes peut également être à l'origine de dommages cellulaires. Des études menées chez *M. mycoides* subsp. *mycoides* SC ont montré que les souches africaines possédaient plusieurs gènes impliqués dans le transfert du glycérol (GtsABC), contrairement aux souches européennes considérées comme moins virulentes. Cette incapacité à transporter le glycérol pourrait être à l'origine de l'atténuation de leur virulence (Vilei et Frey, 2001). Le glycérol absorbé serait phosphorylé en glycérol-3-phosphate (G3P) puis la (L-alpha)-glycérophosphate oxydase (GlpO), protéine membranaire, catalyserait l'oxydation du G3P en dihydroxyacétone phosphate (DHAP) avec production de peroxyde d'hydrogène (H_2O_2). Les souches européennes, incapables de transporter le glycérol, produisent une quantité réduite d'H_2O_2 et sont moins cytotoxiques que les souches africaines (Vilei et Frey, 2001; Pilo *et al.*, 2005; Bischof *et al.*, 2008).

La production d'H_2O_2 a été rapportée pour certaines souches de *M. agalactiae* (Khan *et al.*, 2005).

Perturbations du cycle cellulaire

Les mycoplasmes peuvent se développer en culture cellulaire et interagir avec les cellules eucaryotes durant de longues périodes sans induire d'effets cytopathiques aigus. Cependant, la présence de mycoplasmes à la surface ou à l'intérieur des cellules eucaryotes peut induire des signaux capables de perturber le cycle cellulaire.

Des études récentes ont montré que plusieurs mycoplasmes peuvent provoquer une inhibition de la prolifération et induire la mort cellulaire accompagnée par une fragmentation de l'ADN caractéristique de l'apoptose. On peut citer *M. arginini*, *M. bovis*, *M. hyorhinis*, *M. penetrans*, *M. fermentans*, *M. mycoides* subsp. *mycoides* SC et *M. alligatoris* (Komada *et al.*, 1997; Paddenberg *et al.*, 1998; Sokolova *et al.*, 1998; Bendjennat *et al.*, 1999; Hall *et al.*, 2000; Dedieu *et al.*, 2005; Hunt et Brown, 2005, 2007). Paradoxalement, en plus de leur effet apoptotique, *M. fermentans* et *M. penetrans*, peuvent induire une prolifération cellulaire (Feng

et Lo, 1999; Gerlic *et al.*, 2004, 2007). Des effets mitotiques ont été observés lors de l'infection par *M. arginini*, *M. arthritidis*, *M. fermentans* et *M. penetrans* (Stuart *et al.*, 1990; Feng et Lo, 1994). Enfin, des aberrations chromosomiques et une instabilité génétique ont été décrites en présence de *M. orale*, *M. hominis*, *M. salivarium* et *M. fermentans* (Fogh et Fogh, 1965; Paton *et al.*, 1965; Aula et Nichols, 1967). Les mécanismes responsables de l'altération de la structure des chromosomes restent toutefois inexpliqués.

6. Objectifs et stratégies

Le contrôle des mycoplasmoses est un enjeu important en santé animale. Quelle que soit la filière considérée, les moyens de lutte actuels sont insatisfaisants. Le manque d'efficacité des méthodes prophylactiques et thérapeutiques est en grande partie lié au caractère atypique des mycoplasmes. Les analyses de génomique comparative menées jusqu'à présent ont permis d'obtenir un grand nombre de données *in silico* mais peu de facteurs impliqués dans la virulence des mycoplasmes ont été identifiés. La nature des interactions hôtes-mycoplasmes diffère des schémas déjà décrits chez d'autres bactéries et les stratégies de lutte basées sur les connaissances disponibles se révèlent inadaptées. Le développement d'outils de génomique fonctionnelle ouvre de nouvelles perspectives pour la compréhension des mécanismes du pouvoir pathogène de ces bactéries.

L'objectif général de cette étude est d'identifier les facteurs de virulence des mycoplasmes de ruminants et, plus spécifiquement, les régions génomiques impliquées dans les interactions entre *M. agalactiae* et les cellules de l'hôte.

L'analyse à haut-débit des facteurs impliqués dans la virulence souffre de l'absence de crible de sélection adéquat. En effet, le modèle expérimental sur l'hôte naturel ne permet le criblage que d'un nombre restreint de mutants. Un système de criblage en culture cellulaire a donc été développé pour contourner cette limitation. Ce système a permis d'identifier des facteurs impliqués dans les interactions entre *M. agalactiae* et les cellules de l'hôte et la caractérisation de certains de ces facteurs a été réalisée.

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CHAPITRE 1

PREMIER CHAPITRE : *MYCOPLASMA AGALACTIAE* INTERACTION WITH MAMMALIAN CELLS: THE CRITICAL ROLE OF DISPENSABLE GENES

Eric Baranowski, Sébastien Guiral, Eveline Sagné, Agnès Skapski, and Christine Citti

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1. Résumé

Les mycoplasmes sont des bactéries minimales, dont le génome dépasse à peine la somme d'informations nécessaires à maintenir une vie autonome. Malgré cette apparente simplicité, plusieurs mycoplasmes sont pathogènes pour l'homme et de nombreuses espèces animales chez lesquels ils établissent des interactions étroites avec les cellules épithéliales des surfaces mucosales. Afin identifier les fonctions biologiques impliquées dans les interactions entre les mycoplasmes et les cellules de mammifères, nous avons construit, par mutagénèse transpositionelle, une banque de mutants chez *Mycoplasma agalactiae*, un pathogène de ruminant, et sélectionné les mutants qui présentaient une capacité de multiplication réduite en culture cellulaire. Des réductions variant d'un facteur 3 à une complète inhibition de croissance ont été observées en présence de cellules HeLa. Le site d'insertion du transposon chez les mutants sélectionnés a révélé 18 régions génomiques potentiellement impliquées dans les interactions entre *M. agalactiae* et les cellules HeLa. Plusieurs de ces régions codent pour des protéines avec des caractéristiques de lipoprotéines membranaires. Certaines ont été échangées par transfert horizontal de gènes avec d'autres mycoplasmes de ruminants phylogénétiquement distants de *M. agalactiae*. Deux mutants avec un phénotype extrême contiennent un transposon dans le locus NIF qui code pour des homologues de SufS et SufU, deux protéines impliquées dans la biosynthèse des groupes [Fe-S] chez les bactéries à Gram positif. Des études de complémentation ont confirmé que le locus NIF est indispensable pour la prolifération de *M. agalactiae* en présence de cellules HeLa et d'autres lignées cellulaires de mammifères, alors qu'il n'est pas essentiel pour sa culture en milieu axénique. Bien que nos résultats soulèvent des questions concernant les fonctions essentielles chez les mycoplasmes, ils fournissent également un moyen pour étudier leur rôle en tant que pathogènes minimaux.

MYCOPLASMA AGALACTIAE INTERACTION WITH MAMMALIAN CELLS: THE CRITICAL ROLE OF DISPENSABLE GENES

Eric Baranowski[1,2], Sébastien Guiral[1,2,¥], Eveline Sagné[2,1],Agnès Skapski[1,2] , and Christine Citti[1,2,*]

[1] INRA, UMR 1225, F-31076 Toulouse, France

[2] Université de Toulouse, ENVT, UMR 1225, F-31076 Toulouse, France

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Running title: Essential genes of *M. agalactiae* in cell culture

*** Corresponding author:**

Christine Citti

UMR1225 INRA, ENVT

Ecole Nationale Vétérinaire de Toulouse

23 chemin des Capelles, BP 87614

31076 Toulouse cedex 3, France

Phone: +33 5 6119 38 56 Fax: +33 5 6119 3273

Email: c.citti@envt.fr

[¥] Present address: Institut de Biologie et Chimie des Protéines, CNRS, Université de Lyon, 7, passage du Vercors, 69367 Lyon Cedex 07, France

2. Abstract

Mycoplasmas are minimal bacteria, whose genome barely exceeds the smallest amount of information required to sustain autonomous life. Despite this apparent simplicity, several are successful pathogens of man and animals in which they establish intimate interactions with epithelial cells at mucosal surfaces. To identify biological functions mediating mycoplasma interactions with mammalian cells, we produced a library of transposon knock-out mutants in the ruminant pathogen *M. agalactiae* and used this library to identify mutants displaying reduced multiplication capacity in cell culture. *M. agalactiae* mutants displaying a 3-fold reduction in CFU titers to nearly complete extinction in co-culture with HeLa cells were identified. Mapping of transposon insertion sites revealed 18 genomic regions putatively involved in *M. agalactiae* interaction with HeLa cells. Several of them encode proteins with features of membrane lipoproteins and/or were involved in horizontal gene transfer with phylogenetically distant pathogenic mycoplasmas of ruminants. Two mutants with the most extreme phenotype carry a transposon in the NIF locus which encodes homologues of SufS and SufU, two proteins presumably involved in [Fe-S] cluster biosynthesis in Gram-positive bacteria. Complementation studies confirmed the conditional essentiality of the NIF locus that was found critical for proliferation in presence of HeLa and several other mammalian cell lines, while dispensable for axenic growth. While our results raised questions regarding essential functions in mycoplasmas, it also provides a means for studying their roles as minimal pathogens.

3. Introduction

Often portrayed as minimal bacteria, mycoplasmas have evolved from low G+C content Gram-positive ancestors by massive losses of genetic material and extensive genome downsizing (37, 44). As a consequence of this reductive evolution, mycoplasmas are lacking a significant number of biological functions found in more complex bacteria and have increased their dependence on the host for many nutrients. The absence of a cell wall, a small size, a fastidious growth in cell-free environments and complex requirements for nutrients are among the most emblematic features of these particular organisms (37). Their minute genome which for some species is close to the minimal requirements for sustaining autonomous life is used as an experimental platform to explore the concept of a minimal cell, and as a model system for the design of synthetic bacterial genomes (17, 18, 25, 35). While significant progress has been made in understanding the biology of these minimal organisms in laboratory conditions, little is known regarding mycoplasma factors involved in virulence and host interaction. Recent genomic studies indicated that several mycoplasma species have retained sexual competence, a trait that may provide some pathogenic species with a high potential for adaptation (44, 45).

Mycoplasmas are widely distributed in nature, and several species are successful pathogens capable of establishing persistent infections and causing debilitating diseases in human and a wide range of animal species (37). Mycoplasmas are also recurrently found associated with cultures of mammalian cells where they can survive for long periods, often without apparent signs of contamination but with potential consequences for the reliability of experimental results and the safety of biological products. Classified by the World Organization for Animal Health (OIE) as notifiable diseases, a number of mycoplasma infections in domestic animals can have a significant impact on livestock production (16). Among those, the ruminant pathogen *M. agalactiae* is the main etiological agent of contagious agalactiae in sheep and goats, a syndrome that is characterized by mastitis, arthritis, keratoconjunctivitis and pneumonia (3). Although phylogenetically distant from *M. agalactiae*, several members of the so-called mycoides cluster, such as *M. mycoides* subsp. *mycoides* Large Colony and *M. capricolum* subsp. *capricolum*, are also able to induce similar symptoms in the same ruminant species. Remarkably, whole-genome sequence analysis have revealed that extensive horizontal gene transfer (HGT) events, affecting up to 18 % of *M. agalactiae* genome, occurred between *M. agalactiae* and members of the mycoides cluster, illustrating the unexpected plasticity and adaptability of the mycoplasma genome (44, 45).

Recent advances in whole-genome sequencing have greatly facilitated the study of mycoplasmas (2, 33, 34, 44, 45). Unfortunately, these data alone have been of little help in identifying the mechanism underlying mycoplasma diseases. The main reason is that

mycoplasma predicted gene products, other than those involved in house-keeping functions, display little or no homology to those known for classical bacteria (45). Among the few exceptions is the ADP-ribosylating cytotoxin found in the human respiratory pathogen *M. pneumoniae* that displays some similarity with the pertussis toxin (27).

Factors that may contribute to the pathogenic process in mycoplasmal infections include: the capacity to adhere and invade host cells, the production of immunomodulatory molecules and a highly variable antigenic structure, as well as the formation of biofilm and the release of metabolic hydrogen peroxide (5, 10, 22, 29, 41). Gene products presumably involved in *M. agalactiae* host interaction include the P40 adhesion protein (13), a family of phase-variable surface proteins, designated as Vpma, which are encoded by a locus subjected to high-frequency DNA rearrangements (19, 20), and the immunomodulatory P48 protein (40).

Transposon mutagenesis has been used extensively as a tool for the identification of virulence genes in pathogenic bacteria. Similar approaches have been developed with a few mycoplasma species, mainly with the aim of defining the minimal set of essential genes required to sustain autonomous life in axenic conditions (12, 15, 18, 25). However, the genetic information necessary to develop mycoplasma interactions with its animal host is likely to differ from the minimal set of essential genes required for laboratory growth. New opportunities to investigate mycoplasma factors involved in host interaction have emerged through the development of genomic tools facilitating the manipulation of animal mycoplasmas including *M. agalactiae* (8, 9). However, *in vivo* screening of mutant libraries of ruminant mycoplasmas is faced with the difficulties inherent to experimental infections using large animals.

The present study combines large-scale transposon mutagenesis of the pathogen *M. agalactiae* and a suited model of bacteria-HeLa cells interactions in co-culture. Genomic regions of *M. agalactiae* specifically required for survival under cell culture conditions, while dispensable for axenic growth, were thus identified, indicating that mycoplasma co-cultivation with mammalian cells represent an original and efficient system for high-throughput screening of large mutant libraries.

4. Materials and methods

Bacterial strains and culture conditions

M. agalactiae reference strain PG2 (45) was cultured at 37℃ in Aluotto or SP4 medium (46) supplemented with 500 µg/ml cefalexin (Virbac). Gentamicin (50 µg/ml) was added to the media for the propagation of *M. agalactiae* mutants generated by transposon mutagenesis. Mycoplasma cultures were stored at -80℃, as 10 µl aliquots. CFU titers were determined by serial dilutions in Dubelcco's phosphate-buffered saline (Invitrogen) supplemented with 1 % heat inactivated horse serum (Invitrogen). Dilutions were spotted (10 µl) onto solid Aluotto or SP4 medium, and mycoplasma colonies were counted after 2 to 5 days incubation at 37℃. *Escherichia coli* DH10B [genotype: F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lac*ZΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara, leu*) *7697 galU galK rpsL endA1 nupG*] (Invitrogen) was used for DNA cloning and plasmid propagation. *E. coli* were grown in Luria broth supplemented with ampicillin (50 µg/ml) and/or tetracycline (5 µg/ml) when required. Sheep and horse erythrocytes were derived from defibrinated blood (BioMérieux).

Plasmids and DNA constructions

Plasmid pMT85, which contains a modified version of transposon Tn4001, was kindly provided by Richard Herrmann (48). The gentamicin-resistance marker encoded by the *aacA-aphD* gene is located between the two inverted repeats (IRs) that define the extremities of the transposed fragment, while the transposase gene (*tnpA*) is located outside the transposable elements to prevent re-excision events. Plasmid p20-1miniO/T was used as a shuttle vector for complementation studies in *M. agalactiae*. Plasmid p20-1miniO/T was derived from pMM20-1 (8) by partial deletion of both the *tetM* region and the 6.9 kb DNA fragment carrying the *M. agalactiae* origin of replication. For complementation of *M. agalactiae* mutants T07.082 and T07.134, the NIF locus (MAG0720 and MAG0730) was cloned downstream of the P40 gene promoter region. These two regions were assembled by PCR amplification using an overlapping primer. The promoter region was amplified first by using oligonucleotide primers p40RF-CC (5'-ACGGGGCTAAAGAAGCTGAT-3') and P40-nifS-R (5'-GATCTAATCGATTTAGGCATAATTATTTATATCCTTTTC-3') to generate a 200 bp DNA fragment overlapping CDS MAG0720 at the ATG codon. The NIF locus was then amplified by using the overlapping DNA fragment and primer 88595_R (5'-CTGTGCGCGCTTACAAAGTA-3'). The resulting PCR product was cloned into pGEM-T Easy (Promega), before sub-cloning at the Not I site of the p20-1miniO/T plasmid to generate pO/T-NIF. Cloned sequences were verified by DNA sequencing. PCRs were performed using the Expand High Fidelity PCR System (Roche).

Cell lines

Human epithelial HeLa cells (ATCC CCL2; cervical carcinoma) were kindly provided by P. Mason (University of Texas Medical Branch, Galveston, United States). The bovine turbinate cells (BT; ATCC CRL-1390) were purchased at the ATCC. The bovine cell line KOP (esophageal tissue of a calf) was obtained from the Friedrich Loeffler Institute (Greifswald - Isle of Riems, Germany). The caprine cell lines including SV40-large T immortalized goat embryo fibroblasts (TIGEF) (11) and similarly immortalized milk epithelial cells (TIGMEC) (31) were kindly provided by C. Leroux (INRA, Lyon, France). Cells were grown in DMEM-based medium composed of high glucose (4.5 g/L), sodium pyruvate and GlutaMAX-I containing Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with non-essential amino acids (NEAA; Invitrogen) and 10 % heat inactivated fetal calf serum (FCS; Eurobio). Cells were incubated at 37℃ in an atmosphere with 5 % CO 2, and sub-cultured every 2 to 3 days by seeding one-third to one-sixth the number of cells reached at confluence.

Co-cultivation of *M. agalactiae* with mammalian cells

Co-cultivations were carried out in DMEM-based medium, supplemented with gentamicin (50 µg/ml) for *M. agalactiae* mutants. Since Aluotto broth (up to 0.1 %) has no apparent toxic effect on mammalian cells (data not shown), mycoplasma inoculums were prepared by direct dilution of frozen mycoplasma cultures in DMEM-based medium. Mammalian cells were prepared by trypsin-EDTA treatment of nearly confluent monolayers. After low speed centrifugation, cells were resuspended in DMEM-based medium, seeded in 24-well plates (Falcon) at a density of $2x10^4$ cells/cm², and inoculated with *M. agalactiae* at different multiplicities of infection (MOI). Mycoplasma and mammalian cells were then allowed to grow at 37℃ under 5 % CO2. At different time post inocu lation, mycoplasma titers were determined by CFU titrations following one cycle of freezing/thawing (-80℃/+37℃) to disrupt mammalian cells.

Transformation of *M. agalactiae* with plasmid DNA

Transformation of mycoplasma cells (10^8 to 10^9 CFU) was performed by electroporation using 1 to 3 µg of plasmid DNA, as described previously (9). Following electroporation, mycoplasma cells were incubated in non-selective SP4 medium for 3 h at 37℃. Cells were then allowed to grow in the presence of the appropriate antibiotic for an additional period of 3 to 12 hours before plating them on selective SP4 agar. Gentamicin and tetracycline were used at a concentration of 50 µg/ml and 2 µg/ml, respectively. Isolated colonies were picked after 4 to 7 days and transformants were subcultured in 1 ml of selective SP4 medium.

Transposon mutagenesis in *M. agalactiae*

Transposon mutagenesis in *M. agalactiae* was carried out using plasmid pMT85. Colonies were collected from independent transformations, and subcultured in 1 ml of selective SP4 medium. Cultures of individual mutants were distributed in 96-well plates, and the pMT85-based library was stored at -80℃. Transposon inser tion sites in *M. agalactiae* chromosome were mapped by sequencing the junction between *M. agalactiae* genomic DNA and the 3'-end of the transposon, using the orientation of the gentamicin resistance gene as a reference. Genomic DNA (5 µg) was sequenced using the BigDye terminator chemistry, and oligonucleotide SG8 (5'-GAGTCAGTGAGCGAGGAAGC-3') as primer. Direct sequencing of genomic DNA was performed at the sequencing facility of the Bio-Medical Research Federative Institute of Toulouse (Toulouse, France).

Identification of growth-deficient mutants in co-culture conditions

A 96-pin replicator (Boekel Scientific) was used for high-throughput screening of the library and the identification of mutants displaying reduced multiplication capacity under cell culture conditions. Co-cultivation of individual mutants with HeLa cells was performed in 96-well plates. Cells were seeded in DMEM-based medium at a density of $2x10^4$ cells/cm², and inoculated with individual mutants using the 96-pin replicator. The volume of the sample transferred by one pin of the replicator was estimated at about 1 µl. After 3 days co-cultivation, plates were submitted to one cycle of freezing/thawing (-80℃/+37℃) and spotted onto solid medium using the 96-pin replicator. The development of mycoplasma colonies was observed after 5 to 10 days incubation at 37℃. Growth-defic ient mutants failed to produce detectable CFU upon co-cultivation with HeLa cells. The presence of viable CFU in culture stocks of *M. agalactiae* mutants were controlled by direct spotting onto solid medium.

PCR-based screening of the mutant library

The detection of mutants with transposon insertion events at specific genomic regions in the whole library was performed by PCR amplification using genomic DNA prepared from individual mutants or mutant pools containing up to 96 individual mutants. Mutant pools were constituted by the addition of a 15 to 30 µl aliquot of each mutant culture. Mutants with a transposon inserted at genomic position 180349 (MAG1540) were identified using the *M. agalactiae*-specific primer 181025_TIG_R (5'-TCTCCACAGGAACAGTTGCTTA-3'), which spans genomic positions 181025 to 181004, and the transposon-specific oligonucleotide SG8 (5'-GAGTCAGTGAGCGAGGAAGC-3') priming at the 3'-end of the integrated transposon

sequence. PCR amplifications (25 µl) were performed according to the recommendations of the Taq DNA polymerase supplier (New England Bioloabs).

Southern blot hybridization

Genomic DNA (1 µg) was digested by Hind III, and hybridization was performed in the presence of DIG-labeled DNA probes as described previously (28).

5. Results

Proliferation of *M. agalactiae* in cell culture depends on HeLa cells for nutrients

To assess the capacity of *M. agalactiae* to proliferate under cell culture conditions, HeLa cells were infected with serial dilutions of mycoplasma cultures and the growth of mycoplasmas over a period of 72 hours was determined by enumerating CFUs. Unless incubated with HeLa cells, *M. agalactiae* was unable to grow in DMEM-based medium alone despite supplementation with fetal bovine serum (Fig. 1A). HeLa cells dramatically enhanced *M. agalactiae* proliferation under cell culture conditions, yet CFU titers were about 100-fold lower than those yielded in axenic media classically used to propagate *M. agalactiae* in laboratory conditions (data not shown). Conversely, *M. agalactiae* had no visible effect on cell monolayer development, at least during the first 72 hours of infection (data not shown). At the end of the incubation period, *M. agalactiae* reached an average titer of ca. 10^7 CFU/ml with a mycoplasma/cell ratio estimated at about 50 to 100 bacteria per cell, regardless of the starting inoculum. This offered the possibility to compare simultaneously a large collection of individual mutants of unknown CFU titers as performed below.

The growth-promoting effect of HeLa cells on *M. agalactiae* was further examined by using a cell culture medium pre-incubated with HeLa cells or supplemented with Aluotto broth. As shown in Fig. 1B, *M. agalactiae* growth was observed under both conditions indicating that a deficiency in essential nutrients, rather than the presence of growth inhibitors, was probably responsible for the absence of proliferation in DMEM-based medium. This suggests that HeLa cells may provide a number of nutrients or growth factors that are required for *M. agalactiae* proliferation under cell culture conditions.

Although human epithelial surfaces are probably not a natural environment for *M. agalactiae*, these results indicate that HeLa cells may provide a useful model system to study basic interactions of mycoplasmas with mammalian cells.

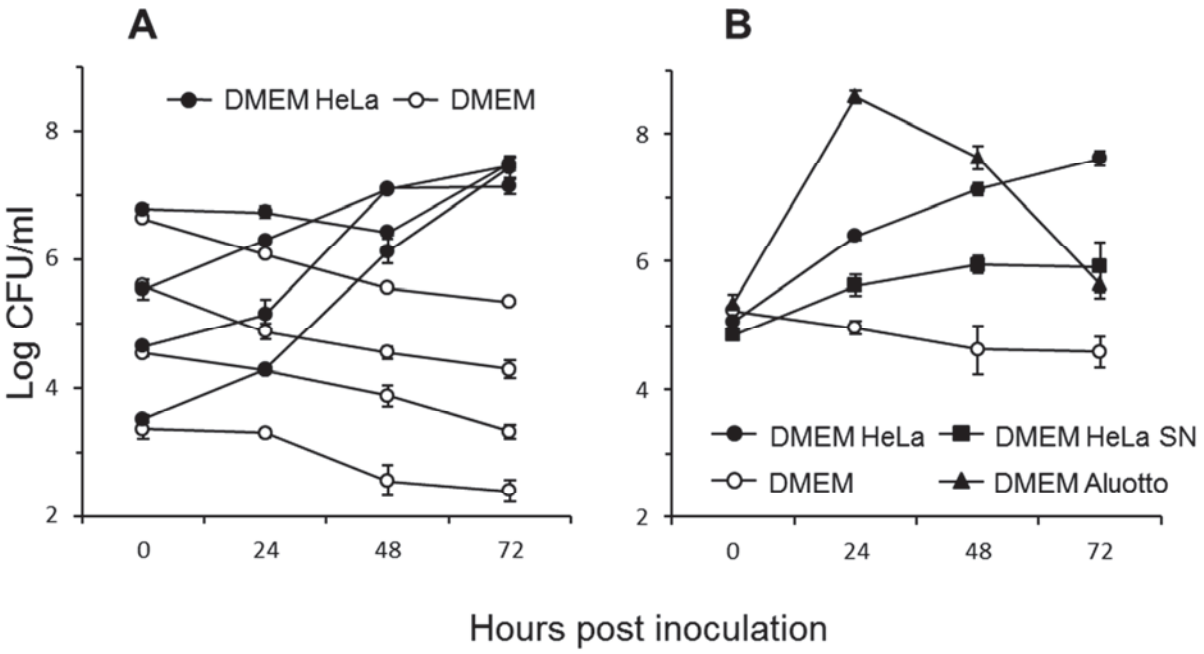


Figure 3: Growth and survival of *M. agalactiae* under cell culture conditions. Serial dilutions from mycoplasma stocks (A) or defined dilutions (B) were inoculated to HeLa cells seeded at a density of $2x10^4$ cells per cm² in DMEM-based medium (DMEM HeLa), and to DMEM-based medium alone (DMEM), DMEM-based medium pre-incubated with HeLa cells (DMEM HeLa SN), or DMEM-based medium supplemented with 10 % Aluotto broth (DMEM Aluotto). Cultures were incubated at 37℃ under 5 % CO_2 and mycoplasma titers (log CFU/ml) were determined by CFU titrations following one cycle of freezing/thawing to disrupt HeLa cells. The data are the mean of at least three independent assays. Standard deviations are indicated by error bars.

Isolation of *M. agalactiae* growth-deficient mutants in cell culture

A *M. agalactiae* library of 1813 gene-disrupted mutants was generated by transposition mutagenesis using plasmid pMT85 (48), which does not replicate in mycoplasma and contains a modified version of transposon Tn4001 (mini-Tn). Because in this plasmid the transposase gene has been placed outside of the transposon, the random insertion of the mini-Tn in the mycoplasma genome is stable in addition to conferring gentamicin resistance. Given the low transformation efficiency of *M. agalactiae*, mutants were collected from multiple individual transformations to produce a representative library (Table 1).

A high-throughput screening strategy was developed to identify mutants displaying reduced multiplication capacity in cell culture. Using this approach, a first set of 23 mutants was selected (group A), whose multiplication in cell culture failed to produce detectable titers (with a detection limit of about 10^4 to 10^5 CFU/ml), and a second set of 176 mutants (group B) displaying apparently reduced, but still detectable, CFU production. The distribution of these mutants obtained from independent transformation events ranged from 0 to 5 % for group A, and from 4 to 21 % for group B (Table 1) of the total amount of transformants.

In this study, only mutants of group A were further examined because of their marked phenotype. After sub-cloning, the growth phenotype of each mutant was determined both under axenic and cell culture conditions (Fig. 2). When compared to the parental strain PG2 or to the control mutant, namely T08.101, these mutants displayed a 3-fold reduction in CFU titers to nearly complete extinction in the presence of HeLa cells, while producing wild-type CFU titers in Aluotto broth. One exception was mutant T05.137, whose multiplication was affected both under cell culture and axenic conditions.

Mapping of transposon insertion sites in *M. agalactiae* growth-deficient mutants

Direct sequencing of genomic DNA from group A mutants revealed single transposon insertion events in each mutant, and identified 19 insertion sites mapping within 15 different coding sequences (CDS), and 3 non-coding regions (NCR) (Fig. 2). CDSs found to be disrupted in group A mutants corresponded to proteins belonging to a broad spectrum of functional categories including chaperones (trigger factor; O-COG0544), nucleotide metabolism (adenine phosphoribosyltransferase, F-COG0503), amino acid metabolism (nitrogen fixation protein NifS, E-COG0520), DNA repair (uvrABC system protein A, L-COG0178), as well as proteins with poorly characterized functions (transport protein SGAT, S-COG3037; esterase/lipase, R-COG0596; GTPase EngC, R-COG1162), and hypothetical proteins (HP) of unknown functions, some of which having features of membrane bound lipoproteins.

Localization of transposon insertion sites in the *M. agalactiae* chromosome of group A mutants failed to reveal any hot spot for the transposition of mini-Tn. However, three mutants (13 % of the total number of selected mutants) were found to have a transposon inserted within a 20-kb locus containing a vestige of an integrative conjugative element ICEA encompassing CDS MAG4060 to MAG3860 in *M. agalactiae* strain PG2 (45). Insertions were found in pseudogene MAG3880 (mutant T06.016) and two NCRs located upstream of MAG4010 (mutant T08.070) and downstream MAG3960 (mutant T05.099). Re-examination of *M. agalactiae* genome using AMIgene software (6) predicted the presence of a short CDS (spanning genomic position 469474 to 469319) found disrupted in mutant T05.099, which was not previously annotated.

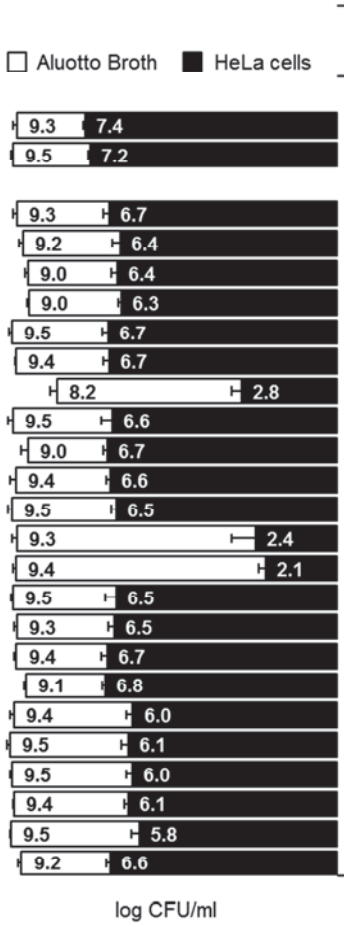
A detailed analysis of transposition events in group A mutants revealed an important bias in the orientation of the integrated mini-Tn sequence. Almost all CDSs found disrupted were harboring a transposon inserted in an antisense direction relative to the transcription of the mutated CDS, using the mini-Tn gentamicin resistance gene as a reference (48). One exception was mutant T07.134 which has a positively oriented mini-Tn in CDS MAG0720 (see below). The *lac* promoter and the promoter of the gentamicin resistance encoding gene are the only regulating sequences provided by the mini-Tn. Both are transcribed in the same direction (48). Experiments are in progress to determine whether transcription from these two promoter regions can extend beyond mini-Tn inserted sequences and influence the orientation of the integrated mini-Tn sequence.

Several growth-deficient mutants identified by our screening strategy were sharing identical insertion sites (Fig. 2). As expected, mutants with identical insertions produced similar CFU titers under co-cultivation conditions, suggesting that these mutants were probably siblings deriving from the same parental clone. To rule out a possible bias in our screening strategy due to the outgrowth of mutants with higher fitness, we used a PCR-based screening method to determine the frequency of mutants sharing identical insertion sites. Analysis of the 149 mutants derived from transformation T05 identified two mutants with a transposon inserted at genomic position 180349 (MAG1540). These two mutants were those selected upon co-cultivation with HeLa cells, suggesting an efficient screening procedure.

Table 1 : Transposon mutagenesis in *M. agalactiae*

Transformation [a]	Mutants [b]	Group A [c]	Group B [d]
T01	52	0 (0.0 %)	5 (9.6 %)
T02	65	0 (0.0 %)	8 (12.3 %)
T05	149	8 (5.4 %)	30 (20.1 %)
T06	169	3 (1.8 %)	35 (20.7 %)
T07	175	2 (1.1 %)	1 (0.6 %)
T08	270	2 (0.7 %)	23 (8.5 %)
T09	247	1 (0.4 %)	47 (19.0 %)
T10	686	7 (1.0 %)	27 (3.9 %)
Total[e]	1813	23 (1.3 %)	176 (9.7 %)

[a] independent transformation assays with plasmid pMT85.
[b] number of clones isolated from each individual transformation assay.
[c] distribution of group A growth-deficient mutants in individual pools of transformants.
[d] distribution of group B mutants (found moderately inhibited upon high-throughput screening of the mutant library in cell culture) in individual pools of transformants.
[e] accumulated values.



Mutant	Genomic position	CDS	CDS position	CDS identity and comments
Parental strain and control mutant				
PG2				Parental strain
T08.101	552034 (-)	MAG4720	0.66 (-)	Control mutant
Group A mutants				
T05.005	699733 (+)	MAG6010	0.24 (-)	HP
T05.081	603060 (-)	MAG5150	0.91 (-)	HP
T05.084	180349 (+)	MAG1540	0.27 (-)	Trigger factor (tig)
T05.094	= T05.084			
T05.098	355485 (+)	MAG2960 *	0.17 (-)	CHP, predicted lipoprotein
T05.099	469389 (+)	NCR		Downstream (39n) of MAG3960 *, CHP; part of ICEA
T05.137	811528 (+)	MAG6960	0.58 (-)	Adenine phosphoribosyltransferase (*apt*)
T05.143	413276 (+)	MAG3480	0.56 (-)	HP
T06.006	706544 (+)	MAG6060	0.36 (-)	HP, predicted lipoprotein
T06.016	459971 (+)	MAG3880 *	0.02 (-)	Pseudogene of CHP, C-terminal part ; part of ICEA
T06.072	754389 (+)	MAG6380	0.91 (-)	Transport protein SGAT
T07.082	087172 (-)	MAG0720	0.18 (-)	Nitrogen fixation protein NifS (*nifS*)
T07.134	088125 (+)	MAG0720	1.00 (+)	Nitrogen fixation protein NifS (*nifS*)
T08.055	004610 (-)	MAG0040 *	0.79 (-)	Esterase/lipase
T08.070	473081 (+)	NCR		Upstream (61n) of MAG4010, HP; part of ICEA
T09.075	450468 (-)	MAG3790	0.95 (-)	uvrABC system protein A (*uvrA*)
T10.168	150813 (+)	MAG1330 *	0.31 (-)	CHP, DUF285 family
T10.277	388694 (+)	NCR		Upstream (79n) of MAG3260 *, CHP, DUF285 family
T10.326	397462 (+)	MAG3350	0.82 (-)	HP
T10.452	245446 (-)	MAG2130	0.75 (-)	GTPase EngC (*engC*)
T10.528	= T10.277			
T10.540	= T10.452			
T10.645	= T10.168			

Figure 4: Characterization of *M. agalactiae* mutants displaying altered growth in cell culture. Mutants were designated according to transformation and clone numbers (e.g. T05.081 designates clone 81 isolated from transformation T05). PG2 and T08.101 refer to the parental strain and the control mutant, respectively. Group A mutants were selected from the mutant library by high throughput screening on HeLa cells, as described in the results section. Mycoplasma titers (log CFU/ml) at 48 hours in Aluotto broth (open bars) and 72 hours in cell culture (solid bars) are compared. The data are the mean of at least three independent assays. Standard deviations are indicated by error bars. Transposon insertion sites were determined by direct sequencing of genomic DNA, and their positions were defined based on the published sequence (NC_009497). The orientation of the inserted sequences is indicated in parenthesis. Mutants sharing an identical insertion are indicated. Coding sequences (CDS) found disrupted in *M. agalactiae* mutants are designated by their mnemonic codification (45). Non-coding regions are indicated (NCR). CDSs involved in horizontal gene transfer between *M. agalactiae* and mycoplasmas of the mycoides cluster (45) are designated by an asterisk (*). For each CDS, the relative position and orientation of the inserted transposon are indicated (CDS position). Hypothetical proteins (HP) have no homolog outside of the *M. agalactiae* species. Conserved hypothetical proteins (CHP) share sequence similarity with proteins of unknown function identified in *Mollicutes* or other bacteria. Several insertion sites mapped within a 20-kb locus containing a vestige of an integrative conjugative element (ICEA).

The NIF locus is essential for *M. agalactiae* growth under cell culture conditions, while dispensable for axenic growth

Two mutants (T07.082 and T07.134) with the most extreme phenotype in co-culture conditions have a transposon inserted in a locus composed of two genes with homology to nitrogen fixation proteins NifS and NifU (MAG0720 and MAG0730). The insertion site identified in mutant T07.082 was found to disrupt the *nifS* gene at a region corresponding to the N-terminal part of the protein (Fig. 2). Mutant T07.134 had a transposon inserted within the same gene, right into the stop codon (TAG). Interestingly, in this mutant, the mini-Tn insertion is such that it restored the sequence of the termination codon suggesting that the phenotype of mutant T07.134 cannot simply be explained by disruption of the gene encoding the NifS protein.

In contrast to the situation described in *M. genitalium* (18, 25), the ability to obtain such mutants suggest that the NIF locus (MAG0720 and MAG0730) is dispensable for *M. agalactiae* growth in axenic conditions. The dispensability of the NIF locus for axenic growth of mutants T07.82 and T07.134 was further confirmed in Aluotto and SP4, two media classically used to propagate *M. agalactiae* in laboratory conditions (data not shown). BLAST analysis of the *M. agalactiae* genome sequence failed to reveal an additional copy of CDS MAG0720 or the presence of a paralog, ruling out the possibility of an insertion event having inactivated one copy of a duplicated *nifS* gene in mutants T07.82 and T07.134. However, essential genes can also be found disrupted if gene products are supplied by other mutants in mixed populations. To rule out this hypothesis, the presence of wild-type sequences in cloned *nifS* mutants propagated in media without gentamicin was tested by PCR assay using oligonucleotide primers flanking the transposon insertion site in mutants T07.82 and T07.134 (Fig. 3). The absence of wild-type sequences was confirmed in all populations tested. Finally, southern blotting analysis performed on *nifS* mutant populations at passage 1 to 20 in selective or non-selective media confirmed the stability of the inserted sequences (Fig. 3).

The growth phenotype of *nifS* mutants T07.82 and T07.134 under cell culture conditions was further characterized. Growth experiments were carried out using HeLa cells and a number of animal cell lines derived from ruminant species (see Materials and Methods). Incubation with HeLa cells, while producing a growth-promoting effect on *M. agalactiae* parental strain and control mutant T08.101, had the opposite effect on *nifS* mutants T07.82 and T07.134 (Fig. 4). This dual effect, growth-promoting versus death-inducing, was not restricted to HeLa cells, and was also observed with all mammalian cell lines tested in this study, although at different degree (Fig. 4). These results suggest that components, most likely nutrients released by mammalian cells, were required for *M. agalactiae* proliferation in DMEM-based medium, while toxic for the *nifS* mutants. This was further confirmed by reproducing this dual effect in

absence of cells, using a DMEM-based medium pre-incubated with HeLa cells (data not shown).

Finally, disruption of the NIF locus was also found to affect *M. agalactiae* growth in the presence of erythrocytes. Whereas *M. agalactiae* development on blood agar plates produced hemolytic zones surrounding colonies, the development of the *nifS* mutants T07.82 and T07.134 was inhibited in a dose dependent manner by erythrocytes or erythrocyte lysates (Fig. 5), but not by erythrocyte ghosts (data not shown). This inhibition was not species specific, since similar results were obtained using horse or sheep erythrocytes. Attempts to further characterize the *nifS* mutant growth-inhibiting factors present in erythrocyte lysates failed. These results further illustrate the vulnerability of the *nifS* mutants when exposed to mammalian cells and the critical role played by the NIF locus for *M. agalactiae* survival under these conditions.

Gene complementation studies

Plasmid p20-1miniO/T was used as a shuttle vector for gene complementation studies in *M. agalactiae*. Mutants T07.82 and T07.134 were transformed with the same plasmid in which the NIF locus was introduced (pO/T_NIF). More specifically, the DNA region encompassing the *nifS* and *nifU* genes of PG2 was cloned in p20-1miniO/T downstream of the P40 protein promoter region (see Materials and Methods). The growth of mutants T07.82 and T07.134 on blood agar plates was restored upon transformation with construction pO/T_NIF, but not with control plasmid p20-1miniO/T (Fig. 5). Complementation of these two mutants was also confirmed in co-culture with mammalian cells (Fig. 5). Growth experiments failed to reveal differences between the parental strain PG2 and complemented mutants. As expected, the phenotype displayed by mutants T07.82 and T07.134 under co-cultivation conditions remained unchanged following transformation with the control plasmid p20-1miniO/T. Complementation studies confirmed the conditional essentiality of the NIF locus for M. agalactiae in all these specific culture conditions.

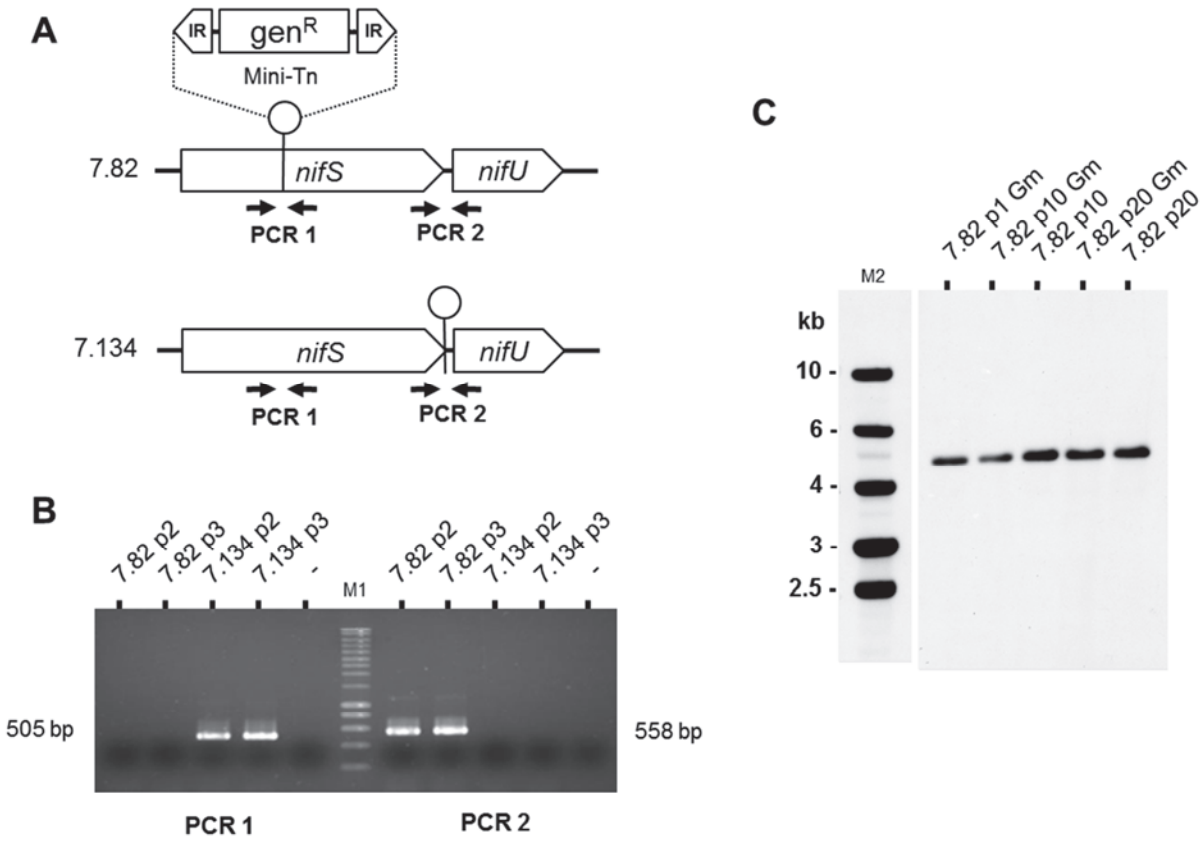


Figure 5: Clonality and stability of *M. agalactiae* NIF mutant populations. (A) The genomic region surrounding the transposon insertion site in mutants T07.82 and T07.134 were analyzed by PCR amplification to detect the presence of contaminating transposon-free sequences. PCR1 and PCR2 were performed using primers pairs 86768F (5'-TCAGCCGACATTATTCATGG-3') - 87272R (5'-CACCGGCTTTTAATTTTTGC-3') and 88037F (5'-AGGGTTTCGCTAGGGGTTTA-3') - 88595R (5'-CTGTGCGCGCTTACAAAGTA-3'), respectively. (B) The PCR1 product (505 bp) amplified from mutant T07.134 populations at passage 2 (7.134p2) and 3 (7.134p3) was not detected upon amplification of the corresponding populations of mutant T07.82 (7.82p2 and 7.82p3). The opposite result was observed for the PCR2 product (558 bp). These negative results suggest the absence of detectable contaminating sequences in all the populations tested. M1, molecular weight markers (SmartLadder; Eurogentec). (C) Southern blot analysis of genomic DNA derived from NIF mutant T07.82 (7.82) at passage number 1, 10 and 20 in selective (Gm) or non-selective medium. Genomic DNA was digested by HindIII and Southern blot hybridized with DIG-labeled amplicons derived from plasmid pMT85. M2, molecular weight markers (1 Kb DNA ladder; Promega).

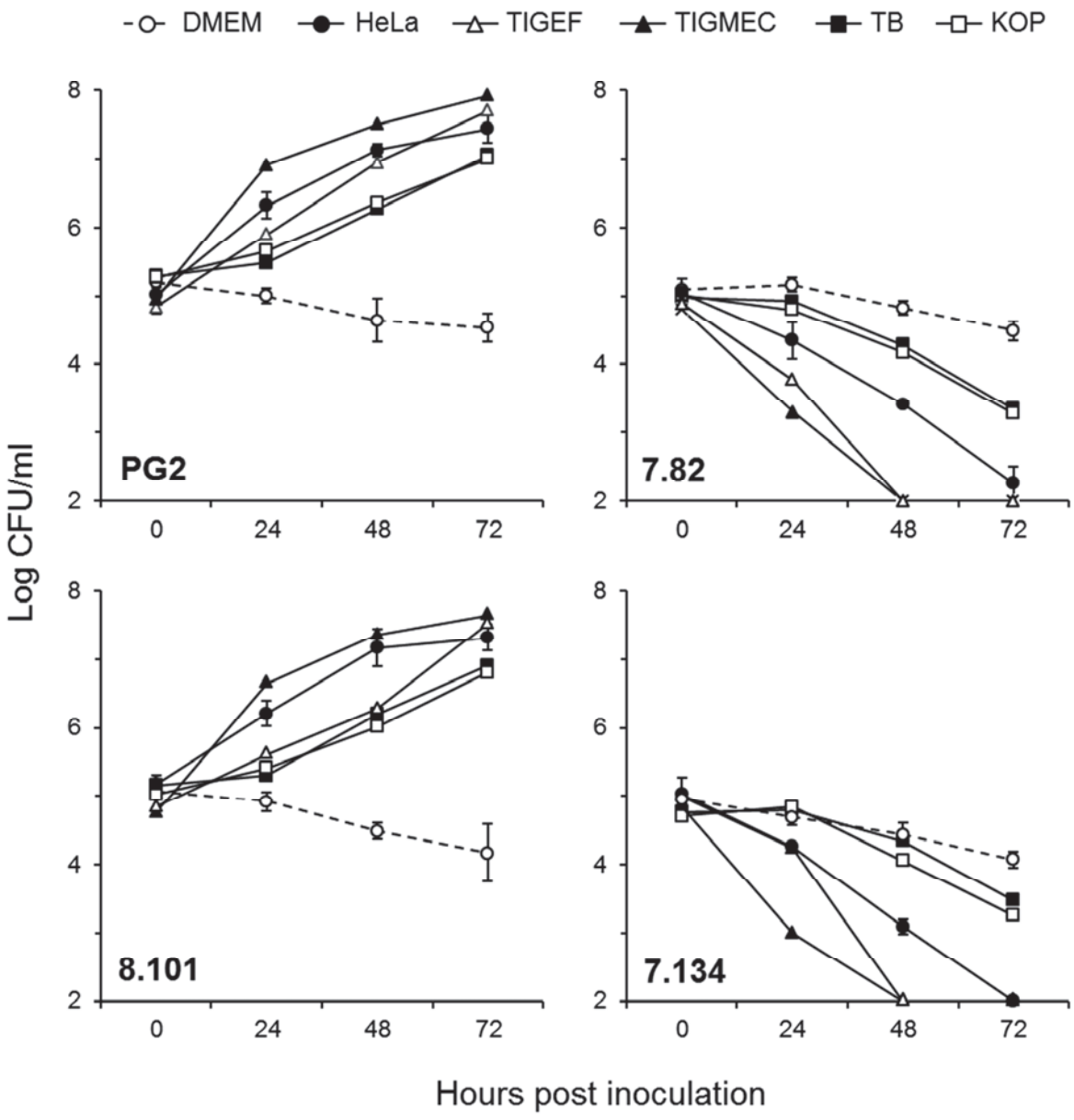


Figure 6: Comparative growth of *M. agalactiae* NIF mutants under cell culture conditions. *M. agalactiae* parental strain PG2 (PG2), the control mutant T08.101 (8.101), and NIF mutants T07.082 (7.82) and T07.134 (7.134) were assessed for survival over 72 hours incubation in DMEM-based medium (DMEM), or in co-culture with a number of mammalian cell lines including HeLa (HeLa), goat fibroblast (TIGEF), goat epithelial (TIGMEC), bovine turbinate (TB) and bovine esophageal (KOP) cells. The data are the mean of two or three independent assays. Standard deviations are indicated by error bars.

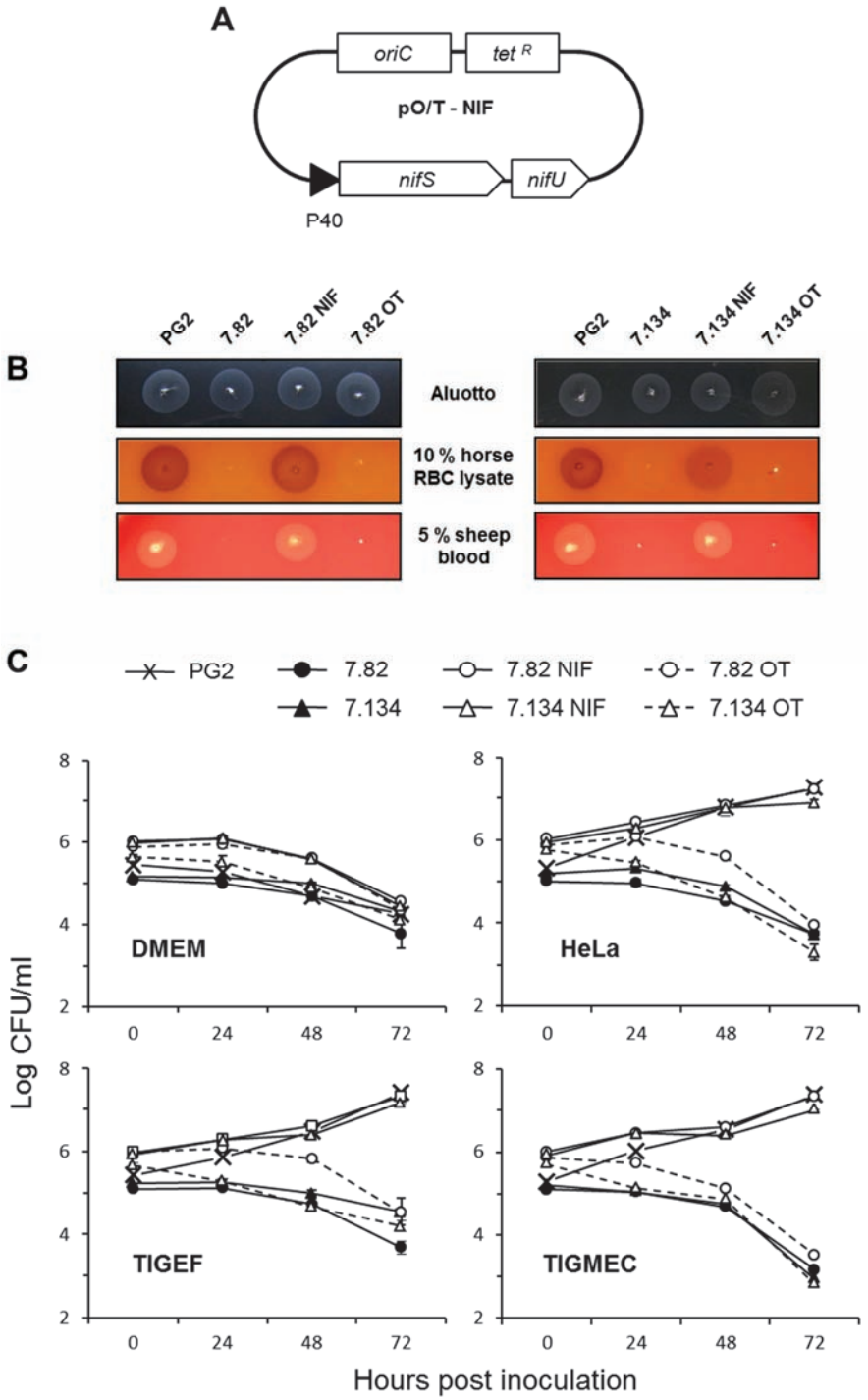


Figure 7: Complementation of *M. agalactiae* NIF mutants. Schematic representation of the plasmid pO/T-NIF used for complementation studies (A). The NIF-locus was cloned under the control of protein P40 promoter region. *M. agalactiae* parental strain PG2 (PG2), mutants T07.082 (7.82) and T07.134 (7.134), and mutants transformed with plasmid pO/T-NIF (7.82 NIF, 7.134 NIF) or the control plasmid p21-1miniO/T (7.82 OT, 7.134 OT) were assessed for colony development on solid Aluotto broth, 10 % horse red blood cells (RBC) lysates or 5 % sheep blood agar plates (B), and for survival over 72 hours incubation in DMEM-based medium (DMEM), or in co-culture with HeLa (HeLa), goat fibroblast (TIGEF) or goat epithelial (TIGMEC) cells (C). The data are the mean of at least three independent assays. Standard deviations are indicated by error bars. Serial dilutions of mycoplasma stocks were spotted on blood agar plates and colony development was observed after 4 to 6 days incubation at 37℃.

6. Discussion

The development of axenic culture conditions has considerably facilitated the study of pathogenic mycoplasmas in laboratory conditions. Yet, limited information is available regarding the factors that are involved in their virulence and in their interaction with the host, mainly because of the lack of cellular or small animal models. In an attempt to fill this gap, a model system using the human epithelial cell line HeLa was developed to study the basic interactions between *M. agalactiae* and eukaryotic cells. Combined with the production of a large mutant library, we further used this model system in a high-throughput screening strategy for the identification of *M. agalactiae* growth-deficient mutants and mapped 18 regions on the *M. agalactiae* chromosome that are specifically required for optimal proliferation under cell culture conditions, while dispensable for propagation in axenic media.

The number of mutants tested was 2.4 times the total number of CDS found in the genome of the PG2 strain (877 kb; 742 CDS). Whether the mutant library produced in *M. agalactiae* may be approaching saturation is unknown, however the number of mutants collected is in agreement with the experimental estimations of the minimal size of a mutant library to reach saturation mutagenesis of all non-lethal insertion sites in other mycoplasma species (15, 18). These estimations were obtained (i) with the human urogenital pathogen *M. genitalium* (580 kb; 475 CDS), for which the number of unique transposon insertion sites drops dramatically after 600 mutants, corresponding to 1.3 times of the total number of CDS found in this organism, and (ii) with the murine pathogen *M. pulmonis* (964 kb; 782 CDS), for which the number of inactivating insertions in genes larger than 1 kb nearly reached a plateau around 1800 insertion sites (2.3 times the total number of CDS). High-throughput screening of the *M. agalactiae* library using the cell system developed here identified a series of 23 mutants displaying a 3-fold reduction in CFU titers to nearly complete extinction in the presence of HeLa cells. The efficiency of this screening was confirmed by (i) the identification of several growth-deficient mutants sharing identical insertion sites such as mutants T05.084 and T05.094 that were present at low frequency (<1.4 %) in the mutant population originating from one transformation event; and (ii) the identification of growth-deficient mutants having a transposon inserted in the same CDS, but at a different position.

The availability of the annotated genome sequence of *M. agalactiae* allowed a rapid mapping of the transposon insertion sites of selected mutants. Disrupted CDSs for which a predicted function was assigned correspond to 40% and belong to a broad number of functional categories, often with no obvious correlation between the predicted function and its potential role in *M. agalactiae* survival under cell culture conditions. Yet, several related functions, such as protein folding, iron-sulfur cluster biosynthesis and DNA repair, have been associated with virulence or stress tolerance in a number of pathogenic bacteria (1, 4, 24, 39, 42, 43, 47).

Another 40% CDSs encode hypothetical products many of which were shown to display membrane lipoprotein features and/or to have undergone horizontal gene transfer (HGT) with the mycoides cluster (Fig. 2). Two of these proteins belong to a gene family, the *drp*, which encodes related proteins containing one or several DUF258 domain. This domain is of unknown function and is found in some bacteria but not in *Mollicutes* with the exception of the mycoides cluster (45). In a recent study comparing *M. agalactiae* strains using whole genomic and proteomic approaches, the differential expression of some *drp* was found in association with the membrane fraction. These data suggested that this family may participate in generating surface diversity, with some *drp* genes presenting features of lipoproteins and being expressed while others serve as sequence reservoir (34). Interestingly, the *drp* genes are part of the gene pool which has undergone HGT with members of the phylogenetically distant mycoides cluster. This cluster only contains ruminant pathogenic mycoplasma species and, in absence a cell-wall, surface exposed lipoproteins may play an important role in mediating host-interaction. Further studies are needed to confirm the role of these CDSs in *M. agalactiae* interaction with mammalian cells, but they provide an interesting subset of mutants that can reasonably be analyzed in vivo. Remarkably, a number of integration events occurred in pseudogenes or in NCRs that map within a particular 20-kb locus containing a vestige of an integrative conjugative element ICEA. The implication of ICEA related ORF in *M. agalactiae* interaction with HeLa cells remains to be confirmed. However it is worth noting that best alignments for ICEA products of the PG2 strain were consistently obtained with *M. capricolum* subsp. *capricolum* ICEC counterparts (45), a member of mycoides cluster which cause similar symptoms. Defining whether the growth-deficiency phenotype observed in selected mutants is due to the single transposon insertion or whether it resulted from phase-variation or spontaneous mutation of other unrelated gene is essential, especially when it concerns regions apparently deprived of CDSs. However, this is hampered by the difficulties in the genetic manipulation of these organisms and, so far, has been performed by complementation with the two mutants that had the most extreme phenotype.

Complementation studies confirmed that key functions conditioning *M. agalactiae* survival and proliferation under cell culture conditions were encoded by the NIF locus. In *M. agalactiae*, the locus is composed of two CDSs encoding homologues of nitrogen fixation proteins NifS and NifU, two proteins involved in iron-sulfur [Fe-S] cluster biosynthesis. Documented in various organisms (14, 26), [Fe-S] cluster assembly systems are poorly understood in Gram-positive bacteria. Recent studies with *Enterococcus faecalis* identified the SUF machinery as the only [Fe-S] cluster biosynthetic system present in the *Firmicutes* genome (38). As expected by the taxonomic position of mycoplasmas, sequence features of the SUF machinery were identified in *M. agalactiae* NifS and NifU proteins. These include the amino acid sequence RSGIFCA

surrounding Cys343 of MAG0720 (NifS) indicative of a group II (SUF-type) bacterial cysteine desulfurases, whose consensus sequence is RXGHHCA, and clearly distinguishes it from group I enzymes of the iron-sulphur cluster, ISC (IscS-type) that display a SSGSAC(T/S)S sequence signature. Similarly, MAG0730 (NifU) product and SufU scaffold proteins share several features that distinguish them from IscU homologues. They both lack the LPPVK motif present in IscU, and contain an 18-21 amino acid insertion between the second and the third conserved cysteine residues. However, despite important sequence homologies with bacterial SUF machineries, mycoplasmas NIF proteins exhibit several unique features.

The NIF locus found present in mycoplasmas is a simplified version of more complex SUF operons and may encode cysteine desulfurases and scaffold proteins with unique biochemical properties. Its strict conservation among all mycoplasma genomes sequenced so far emphasizes its biological importance in *Mollicutes*. Recent studies with a number of pathogenic bacteria, including *Mycobacterium tuberculosis*, *Shigella flexneri*, and the plant pathogen *Erwinia chrysanthemi*, have established a link between [Fe-S] cluster biosynthesis and virulence (24, 32, 39, 42). The central role played by bacterial SUF machineries in resistance to iron limitation and oxidative stress suggest that the NIF locus might play a similar role in *M. agalactiae* -host interactions. However, preliminary in vitro studies with *M. agalactiae* failed to reveal a particular susceptibility of NIF mutants when exposed to oxidative stress or iron limitation. *In vivo* studies are in progress to determine the virulence of the NIF mutants in the animal host. The potential implication of this locus in a broad number of processes involving [Fe-S] proteins considerably increases the functions that can be affected in the NIF mutants. The additional functions that have been attributed to cysteine desulfurases (30), such as the biosynthesis of selenoproteins and multiple cofactors (biotin, lipoic acid, molybdopterin, thiamine, and NAD), as well as iron homeostasis and tRNA modifications, make the situation even more complex.

The growth-deficient phenotype exhibited by the NIF mutants under cell culture conditions but also potentially by other mutants (Fig. 2) unveiled the decisive role played by metabolic functions in the adaptation of *M. agalactiae* to changing environments. A link between carbon metabolism and pathogenicity in mycoplasmas has been already suggested by several groups (5, 21-23, 36). The availability carbon sources *in vivo* and its influence on bacterial pathogenicity has regain interest in the perspective of using carbon metabolic pathways as a viable target for antibiotic development (7). This might be particularly important for mycoplasmas which have limited metabolic capacities and are dependent on the host for many nutrients (37).

Attempts to define the minimal amount of genetic information in various mycoplasma species revealed several discrepancies, since orthologous genes found essential in one organism can

be found dispensable in another. This is illustrated here by the NIF locus which was found essential for axenic growth in *M. genitalium* (18, 25), while apparently dispensable in other mycoplasma species such as *M. pneumoniae*, *M. pulmonis* and *M. agalactiae* (15, 18, 25). A number of situations may account for the occurrence of transposition events in essential genes. The identification of a paralog in *M. pulmonis* of the cysteine desulfurase encoding gene provided a simple explanation for the apparent dispensability of the NIF locus in this species (15). Given the central role played by [Fe-S] proteins in a variety of fundamental biological processes, the absence of a paralog in the *M. agalactiae* genome suggests that essential functions might be performed by unrelated or very distantly related non-orthologous proteins.

The understanding of basic molecular mechanisms underlying cellular life is of broad interest and considerable effort has been devoted in establishing candidate minimal genomes. Our study provides a means for addressing this issue at higher level of complexity, the host-cell context, and new opportunities to decipher mycoplasma host-interaction and virulence.

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CHAPITRE 2

DEUXIÈME CHAPITRE : GENOME-SCALE ANALYSIS OF *MYCOPLASMA AGALACTIAE* LOCI INVOLVED IN INTERACTION WITH HOST CELLS

Agnès Skapski, Marie-Claude Hygonenq, Eveline Sagné, Sébastien Guiral, Christine Citti, and Eric Baranowski

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1. Résumé

Mycoplasma agalactiae est un pathogène important des petits ruminants chez lesquels il provoque l'agalactie contagieuse. Il appartient à un groupe de bactéries minimales qui possèdent un génome de petite taille, des capacités métaboliques limitées et dépendent de leur hôte pour l'apport de nutriments. Bien que la survie des mycoplasmes repose sur des contacts étroits avec les cellules de l'hôte, les facteurs impliqués dans ces interactions ou dans le processus plus général de l'infection sont inconnus. Pour aborder cette question, un système de criblage sur cellules épithéliales et fibroblastes caprins a été utilisé pour l'analyse d'une banque de mutants générée par mutagénèse transpositionelle chez *M. agalactiae*. Des mutants avec une capacité de croissance réduite en culture cellulaire ont été sélectionnés et ont permis l'identification de 62 loci potentiellement impliquées dans l'interaction de *M. agalactiae* avec les cellules de l'hôte. Comme espéré pour des organismes minimaux, la catégorie fonctionnelle « transport et métabolisme » est la plus impliquée dans ces interactions. Cependant, 50 % des gènes interrompus chez les mutants sélectionnés n'ont pas de fonctions connues et codent majoritairement pour des lipoprotéines de surface. Les mycoplasmes sont des bactéries sans paroi et les lipoprotéines, directement exposées à la superficie, sont probablement des acteurs importants des interactions avec l'hôte. Plusieurs régions intergéniques ont également été identifiées et pourraient jouer des rôles régulateurs en conditions de culture cellulaire. De manière intéressante, ce criblage a identifié des groupes de gènes hautement conservés au sein des *Mollicutes*, mais dont la position génomique varie considérablement entre les espèces analysées. L'un de ces groupes de gènes est situé dans une région activement transcrite du génome de *M. agalactiae*, en aval d'un promoteur cryptique. Un scénario possible pour l'évolution de ces loci est discuté. Enfin, plusieurs gènes identifiés au cours de ce criblage sont conservés chez d'autres espèces pathogènes de mycoplasmes. Certains de ces gènes ont été échangés par transfert horizontal entre des espèces phylogénétiquement éloignées. Ces résultats fournissent une base pour l'exploration des interactions hôte-mycoplasme.

GENOME-SCALE ANALYSIS OF *MYCOPLASMA AGALACTIAE* LOCI INVOLVED IN INTERACTION WITH HOST CELLS

Agnès Skapski [1,2], Marie-Claude Hygonenq [1,2], Eveline Sagné [2,1], Sébastien Guiral [1,2 †], Christine Citti [1,2], and Eric Baranowski [1,2*]

[1] INRA, UMR1225, IHAP, Toulouse, France

[2] Université de Toulouse, INP, ENVT, UMR1225, IHAP, Toulouse, France

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*** Corresponding author:**

Eric Baranowski
UMR1225 INRA, ENVT
Ecole Nationale Vétérinaire de Toulouse
23 chemin des Capelles, BP 87614
31076 Toulouse cedex 3, France
Phone: +33 5 6119 32 97 Fax: +33 5 6119 3273
Email: e.baranowski@envt.fr

† Present address: Institut de Biologie et Chimie des Protéines,
CNRS, Université de Lyon, 7, passage du Vercors, 69367 Lyon Cedex 07, France.

2. Abstract

Mycoplasma agalactiae is an important pathogen of small ruminants, in which it causes contagious agalactia. It belongs to a large group of “minimal bacteria” with a small genome and reduced metabolic capacities that are dependent on their host for nutrients. Mycoplasma survival thus relies on intimate contact with host cells, but little is known about the factors involved in these interactions or in the more general infectious process. To address this issue, an assay based on goat epithelial and fibroblastic cells was used to screen a *M. agalactiae* knockout mutant library. Mutants with reduced growth capacities in cell culture were selected and 62 genomic loci were identified as contributing to this phenotype. As expected for minimal bacteria, “transport and metabolism” was the functional category most commonly implicated in this phenotype, but 50% of the selected mutants were disrupted in coding sequences (CDSs) with unknown functions, with surface lipoproteins being most commonly represented in this category. Since mycoplasmas lack a cell wall, lipoproteins are likely to be important in interactions with the host. A few intergenic regions were also identified that may act as regulatory sequences under co-culture conditions. Interestingly, some mutants mapped to gene clusters that are highly conserved across mycoplasma species but located in different positions. One of these clusters was found in a transcriptionally active region of the *M. agalactiae* chromosome, downstream of a cryptic promoter. A possible scenario for the evolution of these loci is discussed. Finally, several CDSs identified here are conserved in other important pathogenic mycoplasmas, and some were involved in horizontal gene transfer with phylogenetically distant species. These results provide a basis for further deciphering functions mediating mycoplasma-host interactions.

3. Introduction

The term “mycoplasma” is used trivially to describe bacteria belonging to the class *Mollicutes*, which includes the genus *Mycoplama*, as well as several other related genera [1]. These micro-organisms have evolved from a low G+C content Gram positive ancestor by “regressive evolution”, resulting in massive genome reduction [2,3]. As a result, contemporary mycoplasmas lack a cell-wall and are commonly described as the smallest self-replicating organisms, because of the small size of their genome (580 to 1,400 kbp) and the paucity of their metabolic pathways. Mycoplasmas and ureaplasmas live in close contact with animal tissues, probably because of their limited metabolic capacity, a feature that is likely to have increased their dependence on hosts for a number of nutrients [2]. Mycoplasmas occur widely in nature and, despite their apparent simplicity, several species are successful pathogens of animals, in which they establish persistent infections and cause chronic disease [4].

Mycoplasma agalactiae is an important pathogen of small ruminants that causes contagious agalactia (CA), resulting in significant losses in the sheep and goat milk industries [5]. It is classified by the World Organization for Animal Health (OIE) as a notifiable disease and the clinical signs include mastitis, arthritis and kerato-conjunctivitis [5]. Contagious agalactia is also caused by several members of the mycoides cluster, including *M. mycoides* subspecies *mycoides* Large Colony type and *M. capricolum* subspecies *capricolum* [5]. Interestingly, while these mycoplasmas are phylogenetically distant from *M. agalactiae*, detailed *in silico* genomic analyses have revealed that extensive horizontal gene transfer has occurred between *M. agalactiae* and members of the mycoides cluster, and as a result these mycoplasmas may share a number of common cell surface functional domains [3,6]. Phylogenetically, *M. agalactiae* is closely related to *M. bovis* [7], a pathogen of large ruminants that causes clinical signs similar to those of contagious agalactia [8]. For all these ruminant mycoplasmoses, the factors involved in colonization, dissemination and pathogenicity are poorly understood. As a number of genetic tools and genomic data are available for *M. agalactiae* [6,9,10], this species is a useful model for studying the molecular players involved in infectious processes and thus furthering comprehension of pathogenic mechanisms in other mycoplasmas.

A common approach used to identify virulence genes in pathogenic bacteria is based on random transposon mutagenesis [11-13]. In mycoplasmas, this approach has mainly been applied to study the minimal set of essential genes [14-16], but has also been successfully employed in a few cases to identify genes potentially involved in pathogenicity [17], gliding motility and adherence [18,19]. Such an approach is needed to further understanding of *M. agalactiae*, as *in silico* analyses of currently available ruminant mycoplasma genomes has failed to reveal unambiguously loci that might contribute to infection. Indeed, predicted *M. agalactiae* gene products have little to no similarity to virulence factors known in other

bacteria, and 40 % of the coding sequences (CDSs) have been annotated as hypothetical proteins with unknown functions [6]. For *M. agalactiae* and other ruminant species, one limitation of global transposon mutagenesis to identify virulence genes is the absence of a small laboratory animal model of infection, as *in vivo* screening in the natural ruminant hosts is constrained by both technical and ethical problems. To overcome these issues, we developed a method for high-throughput screening of *M. agalactiae* knockout mutants by co-cultivating *M. agalactiae* mutants with HeLa cells [20]. This assay allowed the selection of a number of genomic regions potentially required for growth in HeLa cell cultures, but dispensable in axenic conditions. Human epithelial surfaces are not a natural environment for *M. agalactiae*, so we extended our functional genomic study by using two caprine cell lines that are more relevant to the natural host context: goat mammary epithelial cells, TIGMEC, which are likely to be good targets based on the predilection of *M. agalactiae* for the mammary gland, and goat embryo fibroblasts, TIGEF.

Over 2000 *M. agalactiae* mutants were co-cultured with caprine cells and those showing a significant reduction in their capacity to grow were examined in detail, revealing 62 loci potentially required for propagation in the host environment. The relevance of these loci and the potential role of the genes at these loci in *M. agalactiae*-host interactions were analyzed.

4. Materials and Methods

Bacteria, cell lines and culture conditions

M. agalactiae reference strain PG2 (Refseq NC_009497) [6] was grown in Aluotto or SP4 medium as described previously [20]. Titers were determined by serial dilution in Dulbecco's phosphate-buffered saline (Invitrogen) containing 1% heat-inactivated horse serum (Invitrogen). *E. coli* DH10B (Invitrogen) was used for DNA cloning and plasmid propagation. The human and caprine cell lines used in this study included HeLa cells (ATCC CCL2), SV40 large T-antigen immortalized goat embryo fibroblasts (TIGEF) and similarly immortalized goat mammary epithelial cells (TIGMEC). TIGMEC were derived from milk epithelial cells [21]. Immortalized cells exhibited morphological and phenotypic features of parental milk epithelial cells and expressed cytokeratin, a specific marker of epithelial cells. Immortalized goat embryo fibroblasts were generated from carpal synovial membrane explants and displayed morphological features of fibroblastic cells [22]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM)-based medium, as described previously [20], composed of DMEM (high glucose, sodium pyruvate, and GlutaMAX-I; Invitrogen) supplemented with non-essential amino acids (NEAA; Invitrogen) and 10% heat-inactivated fetal calf serum (FCS; Eurobio).

M. agalactiae knockout mutant library

The library of knockout mutants was produced in *M. agalactiae* reference strain PG2 [20]. Transposon mutagenesis was carried out using plasmid pMT85, which does not replicate in mycoplasmas, but contains a modified version of transposon Tn*4001* (mini-Tn) conferring gentamicin resistance [23]. Mutants were collected from individual transformations to produce a representative library of 2,175 individual mutants. The pMT85-based library was propagated in SP4 medium supplemented with 500 µg cephalexin / ml (Virbac) and 50 µg gentamicin / ml (Invitrogen). Transposon insertion sites in the *M. agalactiae* chromosome were mapped by direct sequencing of the junction between the *M. agalactiae* genomic DNA and the 3' end of the transposon using BigDye Terminator chemistry and oligonucleotide primer SG8 (Table 1). Direct sequencing was performed at the sequencing facility at the Bio-Medical Research Federative Institute of Toulouse (Toulouse, France). The 3' end of the mini-Tn was defined using the orientation of the gentamicin resistance gene as the reference [23]. The Pip, MucB and P40 minus mutants were identified by PCR screening of the mutant library using specific oligonucleotide primers [20]. The mutants NifS1 and NifS2 have been described previously as mutants 7.82 and 7.134, respectively [20]. These mutants had a mini-Tn insertion at genomic positions 86804 (Pip), 88958 (MucB), 281483 (P40 minus), 87172 (NifS1) or 88125 (NifS2). The mini-Tn gentamicin resistance gene in the NifS1, Pip, MucB and P40 minus mutants was

in the opposite orientation compared to the disrupted CDS, but in the same orientation in the NifS2 mutant.

High-throughput screening of *M. agalactiae* knockout mutant library in cell culture

A cell-culture assay was used to screen the *M. agalactiae* knockout mutant library and identify mutants displaying a growth-deficient phenotype [20]. Briefly, cells seeded in 96-well plates were inoculated with cultures of individual mutants using a 96-pin replicator (Boekel Scientific). Growth-deficient mutants were selected based on the titers reached at the end of the co-cultivation period. After one freeze-thaw cycle (-80℃/+37℃), co-cultures were spotted onto solid medium using a 96-pin replicator. The development of mycoplasma colonies was used as a cut-off point (Table 2). Culture stocks (inoculum) of *M. agalactiae* knockout mutants were tested by direct spotting onto solid medium. The titer reached by the wild-type after co-cultivation with HeLa cells [20], TIGMEC or TIGEF cells was not influenced by variations in the initial inoculum size (data not shown). *M. agalactiae* was unable to proliferate in cell-culture medium alone [20].

RNA extraction, RT-PCR amplification and primer extension

Total RNA was extracted from 48 hour cultures of *M. agalactiae* using the TRIzol method (Invitrogen). RNA samples were stored at -80℃. The RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm and by agarose gel electrophoresis. RT-PCRs were carried out with the Access RT-PCR System kit (Promega), using 1 µg of total RNA treated with DNAse (RNase-free DNAse, Promega). Reactions were incubated at 45℃ for 45 min, 94℃ for 2 min, then through 30 cycles of 94℃ for 30 sec, 56℃ for 1 min and 72 ℃ for 30 sec, with a final extens ion incubation of 7 min at 72℃. Amplification products were analyzed by agarose gel electrophoresis. Primer extension was carried out using 15 µg of total RNA, 2 pmol of the labeled oligonucleotide primer 5'-6FAM-NifS1 (Table 1), and 200 units of Superscript III Reverse Transcriptase (Invitrogen). RNAs were denatured by 5 min incubation at 65℃. Reverse transcription reactions were performed at 42℃ for 50 min, followed by enzyme inactivation at 75℃ for 15 min. The cDNAs were treated with RNAse A (Promega) for 30 min at 37℃, ethanol precipitate d and resuspended in 10 µl formamide and 0.4 µl of GS-400HD ROX size standards (Applied Biosystems). Product sizes were analyzed using the ABI3730 sequencer (Applied BioSystems) at the GenoToul genomic platform of Toulouse (France).

A reporter system for the detection of transcriptional promoter sites in *M. agalactiae*

The surface antigen P40 CDS MAG2410 was used as a reporter gene to assess transcriptional promoter activity in *M. agalactiae*. DNA sequences to be tested were cloned upstream of CDS MAG2410 using the plasmid p20-1miniO/T as a vector [20]. The 3' end of the *pip* gene was amplified by PCR using the primers Pip-86804_F and Pip-P40_R (Table 1) to generate a 191 bp fragment overlapping CDS MAG2410. CDS MAG2410 was amplified using Pip-P40_F and CCP40-03 (Table 1) to generate a 1188 bp fragment overlapping the 3' end of the *pip* gene. The two overlapping fragments were assembled by PCR amplification using the primers Pip-86804_F and CCP40-03 (Table 1). The resulting PCR products were cloned into the pGEM-T Easy vector (Promega), before sub-cloning into the *Not*I site of plasmid p20-1miniO/T. PCR amplifications were performed using the proofreading Phusion High Fidelity polymerase (Finnzymes). Before cloning into pGEM-T Easy, 3´-terminal deoxyadenosine residues were added to blunt-ended PCR products by following the A-tailing procedure provided by the supplier (Promega). Cloned sequences were verified by DNA sequencing. CDS MAG2410 alone or with its own promoter sequence was used as negative and positive controls, respectively. PCR amplifications were performed using primers P40_RF_CC and CCP40-03 (positive control), and Pip-P40_F and CCP40-03 (negative control) (Table 1).

A surface antigen P40-knockout mutant (P40 minus; see above) was used to test the expression of P40 from the different plasmid constructs. *M. agalactiae* cells (10^8 to 10^9 CFU) were transformed by electroporation using 1 to 3 µg of plasmid DNA, as described previously [20]. After 3 hours incubation in non-selective medium, cells were allowed to grow in the presence of appropriate antibiotic for 24 hours before plating on selective solid medium. Transformants were picked after 4 to 7 days and subcultured in selective SP4 medium. The expression of *M. agalactiae* surface antigen P40 was tested by Western blotting.

Western blotting and immunodetection of *M. agalactiae* lipoprotein P40

Mycoplasmas grown in SP4 medium were collected by centrifugation at 10,000 x g and resuspended in Dulbecco's phosphate-buffered saline (Invitrogen). The protein concentration was determined using the Quick Start Bradford protein assay (Bio-Rad). For Western blotting, total proteins (0.5 µg) were separated by SDS-PAGE using the Mini-Protean II electrophoresis system (Bio-Rad) and transferred to Protran nitrocellulose membranes (Whatman). Membranes were blocked in Tris-buffered saline (TBS) (10 mM TrisHCl, pH 7.4; 150 mM NaCl) containing 5 % skim milk for 2 hours, then incubated overnight at 4℃ with a sheep anti-

P40 serum at a dilution of 1/500 in TBS containing 0.05% Tween 20 and 10% heat-inactivated horse serum (Invitrogen). Western blots were developed using horseradish peroxidase conjugated secondary antibody raised in rabbits (DAKO) and 4-chloro-naphthol as substrate. Sheep serum raised against the *M. agalactiae* surface antigen P80 was used as a control (dilution of 1/200). The anti-P40 and anti-P80 sheep sera were produced by animal immunization with P40 or P80 recombinant proteins, respectively (data not shown).

Table 1. Oligonucleotides used in this study

Name	Sequence (5'-> 3')
SG5	TTTTACACAATTATACGGACTTTATC
SG8	GAGTCAGTGAGCGAGGAAGC
P40_RF_CC	ACGGGGCTAAAGAAGCTGAT
CCP40-03	TGGTTATATTTTCCATATCTTTC
Pip-P40_F	GCAATTGAGAATTTTATTAAAGGATAAATA-ATGAAAACAAATAGAAAAATATTGTTTGGT
Pip-P40_R	ACCAAACAATATTTTTCTATTTGTTTTCAT-TATTTATCCTTTAATAAAATTCTCAATTGC
Pip-86804_F	GCCAGCCATATGGTGCATATTTAG
Pip_F	TATTCGACCAAAGAGGGTGT
Pip_R	TCATCAAAATCACCACCAAG
NifS_F1	TCAGCCGACATTATTCATGG
NifS_R1	CACCGGCTTTTAATTTTTGC
NifS_F2	TGTCACAAAGTTGGAGCAAT
NifS_R2	ACGAAGGAATCGTTACAAGC
NifU_F	AGGGTTTCGCTAGGGGTTTA
NifU_R	CTGTGCGCGCTTACAAAGTA
5'-6FAM-NifS1 [a]	TAGTTCTTGTGCTAACCGAATA

[a] Oligonucleotide labeled by a 6-carboxyfluorescein (FAM) 5'-modification.

5. Results and Discussion

High-throughput identification of *M. agalactiae* growth-deficient mutants upon co-cultivation with host cells

Our group has previously reported the construction of a library of *M. agalactiae* knockout mutants together with the development of a high-throughput screening method based on the co-cultivation of *M. agalactiae* mutants with HeLa cells [20]. Since human epithelial cells are not a natural environment for *M. agalactiae*, we subsequently screened the library with two caprine cell lines, which are likely to be more relevant to the normal host-cell interactions involved in infections with this pathogen. The goat mammary epithelial cells, TIGMEC, are likely to be particularly relevant given the predilection of *M. agalactiae* for the mammary gland. HeLa cells were used as a control.

The ability of individual mutants to grow in the presence of these three cell lines was assessed after 3 days of co-cultivation by directly spotting the cultures onto solid medium and comparing the titers (see Materials and Methods). A total of 209 growth-deficient mutants were selected out of 2,175 tested, using a cut-off value of 10 colonies per plate, which corresponded to a titer of 10^5 CFU/ml in co-cultures (Table 2). The mutants unable to grow in each cell line are shown in Fig. 1A, with a detailed description of those unable to grow on caprine cells provided in Tables 3 and 4. Some differences were seen between the repertoire of mutants identified as growth deficient on HeLa cells in the current study and those identified in our previous study [20], with only 61% common to both studies. This reflects the limitations of these assays, including some cross contamination between individual mutants stored in 96-well plates. These were found to hamper the selection of mutants with reduced growth capacities in cell culture (data not shown) and to affect the reproducibility of the screening from one experiment to the other.

Some mutants (27 %) displayed reduced growth capacities on multiple cell types, while the majority (73 %) exhibited this phenotype on one cell line (Fig. 1A). Interestingly, only a small number of mutants were selected on TIGMEC, compared to the number selected on TIGEF and HeLa cells (Table 2). It is possible that the tolerance of *M. agalactiae* to transposon mutagenesis increases when grown on a cell type similar to its natural environment, but this hypothesis needs to be confirmed with a larger panel of host cell types.

Table 2. High-throughput detection of *M. agalactiae* mutants unable to grow on cultured cells

Cut-off value (CFU titers) [a]	TIGMEC [b]	TIGEF	HeLa
0 (10^4 CFU/ml)	15 (0.7%) [c]	15 (0.7%)	25 (1.1%)
10 (10^5 CFU/ml)	26 (1.2%)	96 (4.4%)	153 (7.0%)

[a] The cut-off value is the number of colonies counted on solid medium following 3 days co-cultivation of *M. agalactiae* knockout mutants with cells. Titers in parentheses indicate the predicted titers in the co-culture.
[b] Number of *M. agalactiae* growth deficient mutants selected on goat mammary epithelial cells (TIGMEC), goat embryonic fibroblast cells (TIGEF), and HeLa cells (HeLa).
[c] Percentage of growth deficient mutants selected from the mutant library.

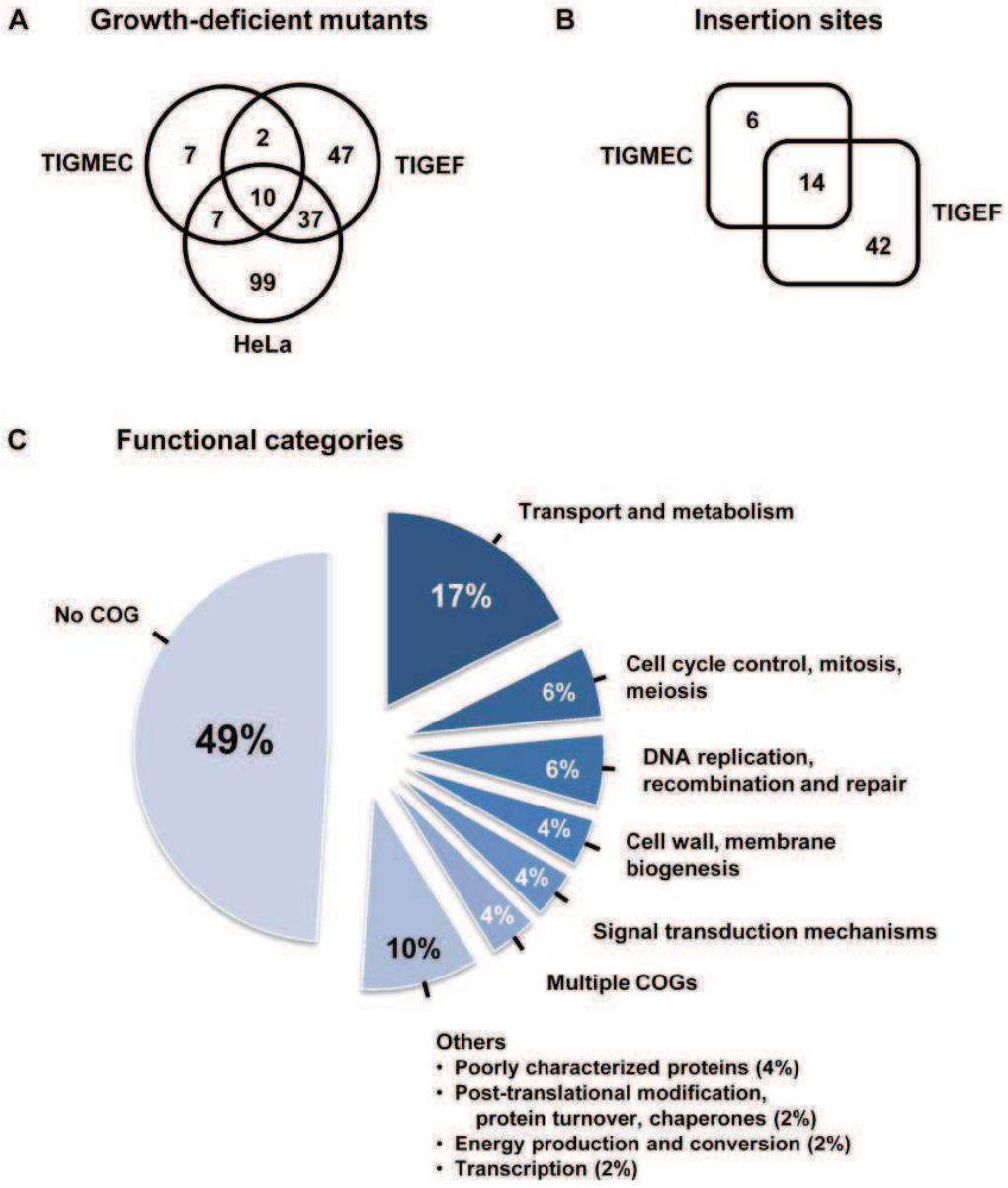

Figure 1. Overall distribution of *M. agalactiae* growth deficient mutants selected after co-cultivation with host cells. (A) Number of mutants selected on goat mammary epithelial cells (TIGMEC), goat embryo fibroblasts (TIGEF) and/or HeLa cells. (B) Number of unique transposon insertion sites identified in mutants selected for their inability to grow on TIGMEC and/or TIGEF. Some mutants had the same insertion site. (C) Distribution in COG categories of the 46 CDSs found disrupted in growth deficient mutants selected on caprine cells (see panel B) [38]. Multiple COG proteins include MAG1490 and MAG2120, which fall into several COG categories (energy production and conversion, C-COG, coenzyme transport and metabolism, H-COG and general function prediction only, R-COG for MAG1490; general function prediction only, R-COG, signal transduction mechanisms, T-COG, transcription, K-COG and replication, recombination and repair, L-COG, for MAG2120).

Mapping of transposon insertion sites in the genome of growth-deficient *M. agalactiae* mutants

Direct genomic sequencing was performed to determine the position of the transposon in mutants found to be inhibited on caprine cell lines. The 110 mutants had 62 unique insertion sites that mapped within 46 different coding sequences (CDS) and 9 non-coding regions (NCR) (Fig. 1B and Tables 3, 4). The examination of DNA sequence chromatograms failed to reveal multiple transposon insertion events in the *M. agalactiae* chromosome (data not shown).

As shown in Figure 1C, most of the disrupted CDSs have been annotated as hypothetical proteins (HP) of unknown function (no COG). Within this category, predicted surface lipoproteins were highly represented 2.1 times more than would be expected [6]. Because of the absence of a cell wall in mycoplasmas, cell surface lipoproteins are thought to be key players in modulating interactions with the host. Interestingly, 20 mutants with a disruption in the same lipoprotein gene (MAG2540) were inhibited on the TIGEF, but not the TIGMEC (Table 3). All had the transposon inserted at the same position, suggesting that they were probably siblings derived from the same parental clone. The repeated selection of this particular mutant suggests a role for MAG2540 in the proliferation of *M. agalactiae* on TIGEF, even though it is dispensable in axenic culture medium and in the presence of TIGMEC. The function of this CDS is unknown, but it is predicted to encode a surface lipoprotein (Vpma-like lipoprotein) with similarities to some domains of the hypervariable Vpma lipoproteins of *M. agalactiae* [24]. Because MAG2540 knockout mutants were also repeatedly selected on HeLa cells, it is possible that, in the absence of specific receptors on target cells such as the TIGMEC, this Vpma-like lipoprotein facilitates binding to ubiquitous structures on mammalian cell surfaces. This implies that growth of *M. agalactiae* in cell cultures requires a close interaction with the cells that is lost in the MAG2540 knockout mutants when growing on TIGEF or HeLa cells. As expected for bacteria with small genomes and reduced metabolic pathways, “transport and metabolism” (COG categories E, F, G, H and P) was the largest functional category involved in interactions between *M. agalactiae* and caprine cells, with 17% of the mutants selected having insertions in genes involved in these functions (Fig. 1C). Since the wild-type is unable to proliferate in cell-culture medium alone [20], it is most likely that these mutants are unable to transport or metabolize some of essential nutrients provided by the cultured cells. The remaining CDSs for which a predicted function had been assigned were distributed across a broad range of functional categories (Fig. 1C), often without any predictable correlation with their role in interactions with caprine cells.

Some of the loci identified in mutants selected on caprine cell lines (Tables 3 and 4), were also identified as being required for growth on HeLa cells, either in this study (Tables 3 and 4)

or in our previous study [20]. This suggests that these loci may be involved in general processes mediating interactions between *M. agalactiae* and mammalian cells. The functions encoded by several of these, including DNA repair (*uvrA*), nucleotide metabolism (*apt*), iron-sulfur cluster biosynthesis (*nifS*), and protein folding (*tig*), contribute to stress tolerance and virulence in a number of pathogenic bacteria [25-31].

A global proteomic analysis of gene products expressed by *M. agalactiae* strain PG2 in axenic culture [32] detected approximately 50% of the CDSs found to be required for growth on caprine cells (Table 3). Whether this reflects the limited sensitivity of the proteomic approach, or differential expression by *M. agalactiae* under axenic and co-culture conditions is not known.

About 20% of the mutants selected on caprine cells had an insertion in a NCR (Table 4). The importance of NCRs in the biology of *M. agalactiae* remains largely unexplored, but several are likely to include transcriptional promoters and other regulatory regions. Re-examination of the 9 mutated NCRs using the AMIGene annotation tool [33] revealed that two, NCR E and G, had an insertion within short CDSs (Table 4) that had not been annotated previously but that could encode products with a role in host-cell interaction. Interestingly, transcriptional activity in NCRs has been reported for a number of mycoplasmas [34,35], suggesting an active role for these regions. NCRs D, E and F (Table 4) are part of a 20 kb locus containing a vestige of an integrative conjugative element (ICEA) [6,36]. The mechanism by which NCRs may regulate the proliferation of *M. agalactiae* in cell culture remains to be elucidated.

Finally, several CDSs required for growth on host cells are conserved in other ruminant mycoplasma species, and/or were involved in the massive horizontal gene transfer (HGT) that occurred between *M. agalactiae*, *M. bovis* and members of the phylogenetically distant mycoides cluster (Table S1). Two CDSs (MAG2870 and MAG6690) were also predicted to have undergone HGT between the avian mycoplasma species *M. synoviae* and *M. gallisepticum* (Table S1). About 70 % of these CDSs involved in HGT encode membrane-associated proteins, and thus are likely to play a role in mycoplasma–host interactions. The genome-scale analysis of *M. agalactiae* in cell culture may assist in understanding pathogenic processes involved in other mycoplasma infections.

Table 3. *M. agalactiae* CDSs identified by high-throughput screening for their reduced growth capacities on cultured cells

CDS name [a]	Cells [b]	No. of mutants (no. of insertion sites) [c]	% CDS (orientation) [d]	Gene	Gene product (COG) [e]	Predicted localization [f]	Detected by MS/MS [g]
MAG0490	F	1 (1)	0.62 (-)		CHP	M	-
MAG0640	F	1 (1)	0.71 (-)	*asnA*	Aspartate-ammonia ligase (E)	C	-
MAG0720	E, F *	2 (2)	0.18 (-) / 1.00 (+)	*nifS*	Cysteine desulfurase (E)	C	-
MAG0890	E, F *	2 (1)	0.85 (+)	*hprK*	Hpr kinase phosphorylase (T)	C	+
MAG1180	E, F *	1 (1)	0.95 (-)	*pepP*	XAA-PRO aminopeptidase (E)	C	+
MAG1330	F *	1 (1)	0.31 (-)		CHP DUF285 family, predicted lipoprotein	M	-
MAG1430	E, F *	6 (3)	0.15 (+) / 0.84 (+) / 0.85 - 0.95 (-)		HP	M	+
MAG1490	F *	1 (1)	0.86 (-)	*ldhD*	D-lactate dehydrogenase (CHR)	IM	+
MAG1500	F	2 (1)	0.56 (+)		Esterase lipase (R)	C	-
MAG1540	F *	1 (1)	0.27 (-)	*tig*	Trigger factor (O)	C	+
MAG1740	F	1 (1)	0.32 (-)	*gidA*	Glucose-inhibited division protein A (D)	C	+
MAG1860	F *	1 (1)	0.63 (-)	*gidB*	Methyltransferase GidB (M)	C	-
MAG1890	F	1 (1)	0.11 (-)		HP	M	+
MAG2110	E	1 (1)	0.08 (+)		Protein phosphatase (T)	C	-
MAG2120	E, F *	1 (1)	0.53 (-)	*pknB*	Serine/threonine-protein kinase (RTKL)	M	+
MAG2540	F *	20 (1)	0.57 (-)		HP, Vpma-like, predicted lipoprotein	M	+
MAG2680	F *	1 (1)	0.60 - 0.70 (-)		HP	M	+
MAG2870	F *	2 (2)	0.16 (-) / 0.72 (+)		CHP, predicted lipoprotein	M	-
MAG2930	E *	1 (1)	0.01 (-)	*atpA*	ATP synthase α chain (C)	IM	+
MAG2960	F *	1 (1)	0.17 (-)		CHP, predicted lipoprotein	M	-
MAG3030	F	3 (1)	0.31 (+)		HP	M	-
MAG3350	F *	1 (1)	0.18 (-)		HP	M	-
MAG3370	F	1 (1)	0.72 (-)		CHP	M	-
MAG3480	F *	1 (1)	0.70 (-)		HP	C	+
MAG3720	E, F	1 (1)	0.10 (+)		CHP	IM	-
MAG3740	F *	1 (1)	0.2-0.4 (+)	*mraZ*	MraZ (S)	IM	-
MAG3790	E, F *	10 (1)	0.95 (-)	*uvrA*	UvrABC system protein A (L)	C	+
MAG3860	F *	1 (1)	ND (-)		CHP	IM	-
MAG4200	F	1 (1)	0.97 (+)		CHP	C	-
MAG4380	F *	3 (2)	0.50 - 0.75 (-) / 0.88 (+)		P115-like ABC transporter ATP binding protein (D)	C	+
MAG4650	E	1 (1)	0.98 (-)		Phosphomannomutase (G)	C	+
MAG4740	F	1 (1)	0.11 (+)		HP, predicted lipoprotein	M	+
MAG4820	E	2 (1)	0.97 (-)		CHP (M)	C	-
MAG4950	E, F *	1 (1)	0.79 (-)		HP, predicted lipoprotein	M	-
MAG5000	F	4 (1)	0.22 (+)		HP	M	-
MAG5150	F *	1 (1)	0.91 (-)		HP, predicted lipoprotein	M	+
MAG5910	F	1 (1)	0.72 (+)		5' nucleotidase, predicted lipoprotein (F)	M	+
MAG6090	F	1 (1)	0.96 (-)		HP, predicted lipoprotein	M	-
MAG6450	E	1 (1)	0.87 (+)		CHP	C	-
MAG6690	F	1 (1)	0.67 (-)		HP	M	-
MAG6760	F	1 (1)	0.07 (-)	*chrA*	Chromate transport protein (P)	M	-
MAG6770	F *	1 (1)	0.99 (-)	*chrA*	Chromate transport protein (P)	M	+
MAG6870	F	2 (2)	0.60 (-) / 0.70 (-)	*dnaX*	DNA polymerase III subunits gamma and tau (L)	C	+
MAG6960	E, F *	1 (1)	0.58 (-)	*apt*	Adenine phosphorybosyltransferase (F)	C	+
MAG7100	F	1 (1)	0.56 (-)	*vpmaZ*	Variable surface lipoprotein D (VpmaZ precursor)	M	+
MAG7200	F	1 (1)	0.74 (+)	*scpB*	Segregation and condensation protein B (K)	C	+

[a] CDS found disrupted in *M. agalactiae* growth-deficient mutants [6].
[b] Letters E and F indicate that the *M. agalactiae* growth-deficient mutants were selected on TIGMEC or TIGEF cells, respectively. Asterisks (*) indicate mutants that were also selected during high-throughput screening on HeLa cells.
[c] For each CDS, the number of mutants identified during the screening on caprine cell lines is indicated, as well as the number of different mini-Tn insertion sites.
[d] For each CDS, the relative position and the orientation of the inserted transposon are indicated. Mini-Tn insertion sites were determined by direct sequencing of genomic DNA, and their positions were defined based on the published genome sequence (NC_009497).
[e] Hypothetical proteins (HP) have no homolog outside the species *M. agalactiae*. Conserved hypothetical proteins (CHP) share sequence similarity with proteins of unknown function identified in other *Mollicutes* or other bacteria. COG categories of encoded proteins are indicated in parentheses [38].
[f] Protein localization was predicted using TMHMM [41]; membrane (M), cytosolic (C), or indirectly linked to the membrane (IM).
[g] Proteins with peptides detected during proteomic analysis of gene products expressed by *M. agalactiae* strain PG2 in axenic culture are identified by a plus sign (+), while proteins not detected are identified by a minus sign (-) [32].

Table 4. *M. agalactiae* NCRs identified by high-throughput screening with host cells

Name [a]	Cells [b]	No. of mutants (no. of insertion sites) [c]	Size of NCR	Genomic position (orientation) [d]	Genetic environment of NCR [e]
NCR A	F	1 (1)	67 nt	31900 (+)	HP (MAG0310) and HP (MAG0320)
NCR B	E, F *	7 (1)	676 nt	388694 (+)	*ptsG* (MAG3250) and CHP DUF285 family (MAG3260)
NCR C	E, F *	2 (1)	740 nt	402664 (+)	HP (MAG3390) and CHP (MAG3400)
NCR D	F	1 (1)	351 nt	460191 (+)	Pseudogene of CHP (MAG3880) and pseudogene of CHP (MAG3890); vestige of ICEA
NCR E	F *	1 (1)	423 nt	469389 (+)	HP (MAG3950) and CHP (MAG3960); AMIGene CDS prediction of 52 AA (from 469474 to 469319); vestige of ICEA
NCR F	F *	2 (1)	330 nt	473081 (+)	HP (MAG4010) and HP (MAG4020); vestige of ICEA
NCR G	E	1 (1)	802 nt	648734 (-)	*gyrA* (MAG5630) and *hsdS* (MAG 5640); AMIGene CDS prediction of 67 AA (from 648894 to 648694)
NCR H	F	1 (1)	290 nt	761860 (-)	HP (MAG6430) and putative prophage protein ps3 (MAG6440)
NCR I	F *	2 (2)	80 nt	843595 (+) / 843634 (+)	*cmk* (MAG7250) and HP (MAG7260)

[a] NCRs were labeled with a letter based on their position in the genome of *M. agalactiae*.
[b] Letters E and F indicate NCRs carrying insertions in mutants with reduced growth capacities on TIGMEC and/or TIGEF, respectively. Asterisks (*) indicate mutants that were also selected during high-throughput screening on HeLa cells.
[c] For each NCR, the number of mutants identified during the screening with caprine cells is indicated, as well as the number of insertion sites.
[d] Transposon insertion sites were determined by direct sequencing of genomic DNA and their positions were defined based on the published genome sequence (NC_009497). The orientation of the transposon is indicated in parentheses.
[e] Surrounding CDS and mini-Tn disrupted AMIGene CDS predictions; CDS names are given in parenthesis; vestige of ICEA indicates an NCR located within a 20 kb locus containing a vestige of an integrative conjugative element (ICEA).

The frequent occurrence of promoter regions in the genome of *M. agalactiae* reduces polar effects mediated by integrated transposon sequences

As mentioned above, some CDSs required for growth on cell cultures fell into functional categories (Fig. 1C) that had no obvious correlation with mycoplasma-host interactions. This raised the question of whether growth-deficient phenotypes can result from a polar downstream effect rather than the effect on the gene disrupted by the transposon insertion. The compact mycoplasma genome, which is often organized into highly dense co-linear gene clusters with operon-like structures, suggests that this might be likely [3].

To evaluate the potential influence of the mini-Tn insertions on gene expression from these operon-like structures, we analyzed the 4 kb co-linear gene cluster *pip-nifS-nifU-mucB* (MAG0710 to MAG0740) (Fig. 2). This cluster was of particular interest because: (i) *nifS* has been shown to be essential for proliferation of *M. agalactiae* on cell cultures, and two knockout mutants, NifS1 and NifS2, were identified by high-throughput screening on caprine and human cells (Table 3); and (ii) these co-linear genes are likely to be co-transcribed by a promoter located upstream of *pip*, based on genome data indicating short intergenic distances (Fig. 2A). Indeed, the operon-like structure of the *pip-nifS-nifU-mucB* cluster was further supported by our transcriptional analyses, which detected overlapping transcripts by RT-PCR (Fig. 2A). Two mutants, Pip and MucB, with a transposon inserted into the corresponding genes, were searched for and found in the mutant library (see Materials and Methods), but both are able to grow on all three cell lines. When tested individually with TIGMEC, both mutants had the wild-type phenotype (Fig. 2B). This result was surprising, at least for the Pip mutant, which was expected to have a similar phenotype to that of the NifSs mutants because of the predicted polar effect of the transposon insertion (Fig. 2A).

RT-PCR amplification using oligonucleotide primers bracketing the mini-Tn integration site in mutants Pip, NifS1, and NifS2 did not detect mRNA (RT-PCR II in mutants Pip and NifS1, and RT-PCR IV in mutant NifS2), but, unexpectedly, transcripts were detected downstream of the mini-Tn (RT-PCR III and IV in mutants Pip and NifS1), suggesting that there may be a promoter within *pip* or the mini-Tn. The presence of a promoter within the integrated mini-Tn sequences was suggested by the identification of RNA transcripts that overlapped the 3' end of the mini-Tn and *nifS* in Pip and NifS1 mutants (data not shown). The extension of transcription beyond the 3' end of the mini-Tn was probably responsible for the expression of downstream genes in knockout mutants of *M. agalactiae* and may have influenced the orientation of the mini-Tn in the mutant library [20]. Although promoter sequences in the mini-Tn remain to be formally demonstrated, constitutive gene expression from the 3' end of the mini-Tn can also be viewed as another outcome of transposon mutagenesis. Experiments are

in progress to determine the role of this mechanism in mutants unable to grow in cell cultures that harbor a transposon within a NCR.

Recent studies on *Mycoplasma genitalium* have revealed that the genome of this human pathogen is actively transcribed, and contains multiple cryptic promoters [34]. This led us to further explore transcriptional activity within the *pip-nifS-nifU-mucB* cluster, and to examine the promoter activity at the 3' end of the *pip* gene. A reporter system was developed to detect promoter sequences in *M. agalactiae* (Fig. 2C). Lipoprotein P40 (MAG2410) is a surface antigen of *M. agalactiae*, the expression of which can be detected using specific antibodies. The successful complementation of a *M. agalactiae* mutant, P40 minus, harboring a transposon within the P40 lipoprotein gene, with a plasmid expressing P40, led us to develop a reporter system to detect promoter activity. Western blotting analyses confirmed that the 157 nucleotides at the 3' end of the *pip* gene, when inserted at the 5' end of the P40 lipoprotein gene, promote complementation of the P40 minus mutant.

Finally, the transcriptional start point at the 3' end of the *pip* gene was mapped by primer extension using the oligonucleotide 5'-6FAM-NifS1 (Table 1). A major extension product with a size of between 163 and 165 nucleotides was obtained, indicating that the transcriptional start point was 31 nucleotides upstream of the stop codon of the *pip* gene (Fig. 2D). The detection of multiple extension products differing in length by a single nucleotide is a common finding in mycoplasma promoters [34,37].

The genomes of mycoplasmas are characterized by a low G+C content. This particular feature may favor the occurrence of primary or secondary promoter regions in the genome of *M. agalactiae*, even within coding sequences such as this promoter within the 3' end of the *pip* gene. The high density of promoters within the genome of *M. agalactiae* may have important evolutionary consequences, facilitating chromosomal rearrangements and gene shuffling in these rapidly evolving organisms.

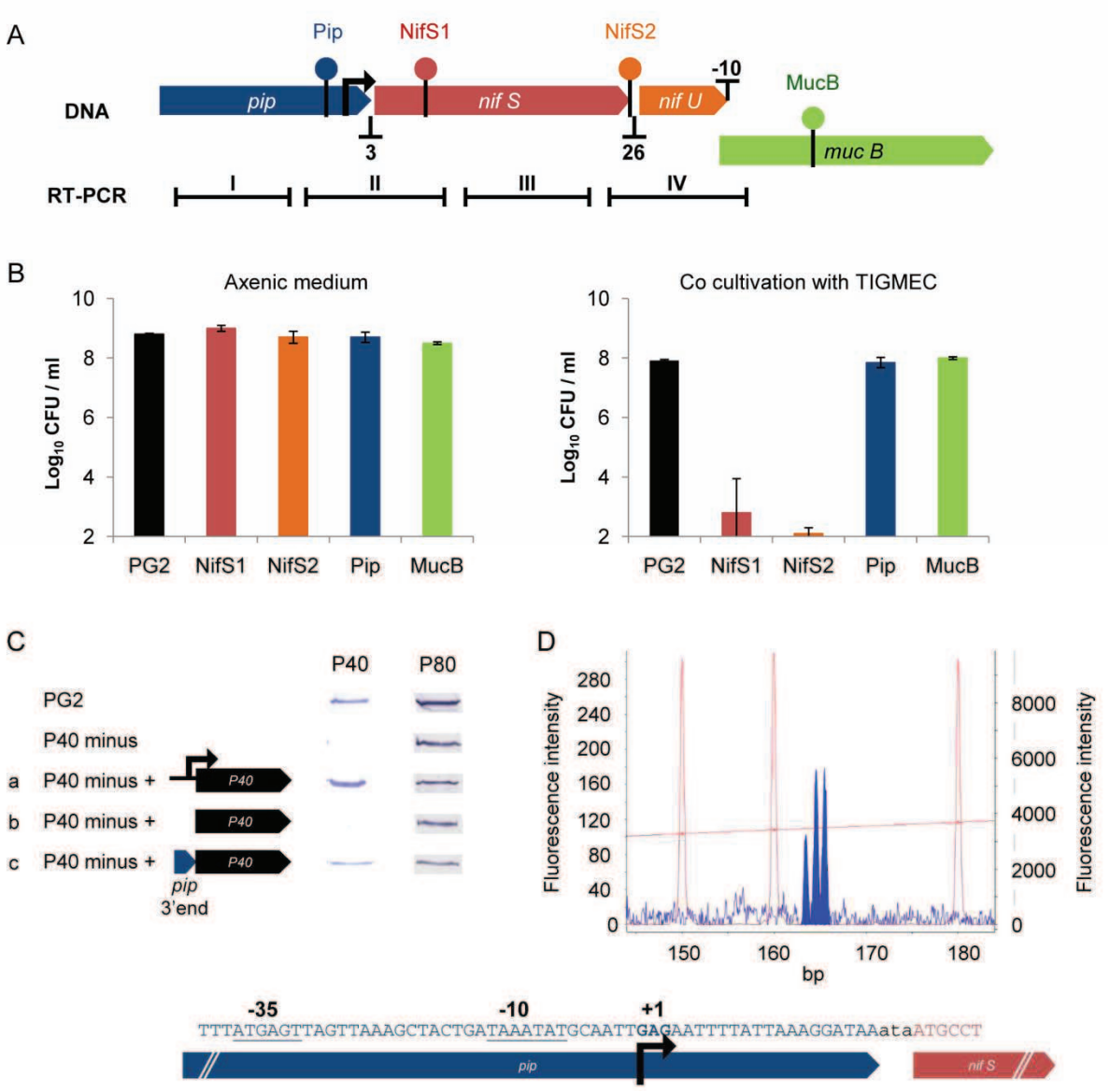

Figure 2. The *nif* locus in *M. agalactiae* is a promoter-less CDS cluster inserted within a transcriptionally active region. (A) Schematic representation of the 4 kbp co-linear gene cluster *pip-nifS-nifU-mucB* indicating the four regions amplified by RT-PCR. Intergenic distances in nucleotides are indicated below. Transposon insertion sites are indicated by filled circles using a different color code for each mutant. RNA regions amplified by RT-PCRs I to IV are indicated by black bars. The promoter identified in the 3' end of *pip* is indicated by a black arrow. (B) Bar graph indicating the titers obtained, after cultivation in axenic media or in the presence of TIGMEC, of *M. agalactiae* strain PG2 or mutants NifS1, NifS2, Pip and MucB. Titers were determined after 48 h cultivation in axenic medium and after 72 h co-cultivation with TIGMEC. The bars indicate the means of three independent assays, with standard deviations indicated by error bars. (C) Identification of promoter sequences in *M. agalactiae* using a new reporter system. The surface antigen P40-knockout mutant (mutant P40 minus) was complemented using plasmid constructs containing the P40 coding sequence preceded by its own promoter (a), without a promoter (b), or preceded by the 3' end of *pip* (c). The expression of the P40 antigen was detected by Western blotting. The surface antigen P80 was used as control. (D) Chromatogram obtained after primer extension of total RNA extracted from a *M. agalactiae* PG2 culture with primer 5'-6FAM-NifS1 (Table 1) as described in the Materials and Methods. Red peaks correspond to the GS-400HD ROX internal lane standards and the shaded blue peaks to the primer extension product. The size is indicated on the X-axis in base pairs. The peak height indicates the fluorescence intensity (arbitrary units) with GS-400HD ROX fluorescence measured on the right Y-axis and FAM fluorescence on the left Y-axis. The position of the transcriptional start point in CDS MAG0710 (*pip*) identified by primer extension experiments is indicated. Putative -10 and -35 regions are underlined. The transcriptional start points are indicated in bold.

Gene involved in the interaction of *M. agalactiae* with host cells may have been hitchhiking across evolution

Although polar effects resulting from the disruption of co-transcribed gene clusters cannot be ruled out, the identification of multiple transposon insertion sites in the same region suggests that important factors mediating the interaction between *M. agalactiae* and host cells may map to these loci. Among the 62 loci identified after screening with host cells, six loci mutants had insertions in the same CDS or NCR, but at different positions (Tables 3 and 4). CDSs with multiple insertion sites included two HP predicted to be exposed on the cell surface (MAG1430 and MAG2870), and cytosolic proteins with homologies with cysteine desulfurase (MAG0720), the P115-like ABC transporter ATP binding protein (MAG4380), and DNA polymerase III subunits gamma and tau (MAG6870). Only one NCR (NCR I) was found containing two different insertions that inhibited the capacity of *M. agalactiae* to grow on host cells. Interestingly, CDSs MAG0720 (*nifS*) and MAG6870 (*dnaX*) each belong to co-linear gene clusters (Fig. 3) that are highly conserved in *Mollicutes* (Fig. S1-3). The remarkable conservation of these two gene clusters led us to examine the co-localization of the 46 CDS identified as being required for growth of *M. agalactiae* on cultured cells, and to compare the synteny of these CDSs using complete genome sequences of mycoplasmas available in public databases. A total of 8 highly conserved co-linear gene clusters were identified (Fig. 3 and Fig. S1-3). Several of these clusters encode proteins predicted to be involved in specific functions, including [Fe-S] cluster biosynthesis (NIF), chromate transport (CHR), cell division (MRAZ) and DNA replication, recombination and repair (DNAX). The potential role of these CDS clusters in the interaction between *M. agalactiae* and host cells was further supported by the similar effect of transposon insertions at different positions in these CDS clusters (NIF, PKNB, MRAZ, CHR and DNAX in Fig. 3). The essential role of the NIF locus in the interaction between *M. agalactiae* and host cells has been demonstrated previously by complementation studies [20].

The location of these CDS clusters can differ considerably from one mycoplasma species to another, even within the same phylogenetic group (Fig. S1-3). In *M. agalactiae*, promoter sequences initiating the transcription of *nifS* were located outside of the NIF locus, with a sequence identified within the 3' end of the *pip* gene (see above). Interestingly, the *pip* gene does not always co-localize with the NIF locus in other mycoplasma species, suggesting that this locus is inserted within transcriptionally active regions. Thus, it is tempting to speculate that some of the genes involved in the interaction between *M. agalactiae* and host cells may have been hitchhiking across evolution for transcriptionally active regions of the mycoplasma chromosome. Further evidence is needed to support this hypothesis.

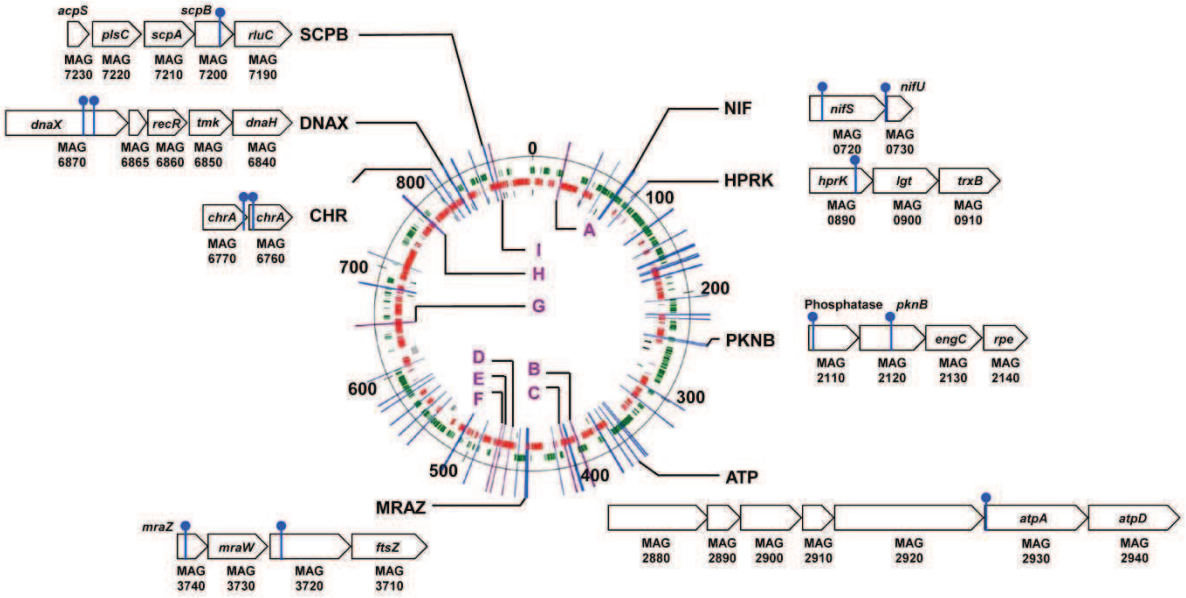

Figure 3. Genomic loci carrying transposon insertions in *M. agalactiae* mutants displaying reduced growth capacities in cell culture. Map of the 62 genomic regions found to be disrupted in *M. agalactiae* mutants selected on TIGMEC and/or TIGEF cells, produced using the Artemis genome browser and annotation tool [39]. Insertion sites found within CDS regions are indicated by a blue bar, while intergenic regions are designated by a letter code and a purple bar. Short CDS clusters are designated by capital letters and a schematic illustration of the locus, in which insertion sites are indicated by a filled circle on top of a blue bar. Genes are labeled. CDSs for hypothetical proteins of unknown function are labeled with their gene number. Genomic distances are indicated in kbp. *M. agalactiae* CDSs are colored in green (positive) or red (negative) on the chromosome to indicate their orientation. Non-coding RNAs are colored in black.

In conclusion, cell culture provides a simple and efficient screening system for genome-scale analysis of mycoplasma loci contributing to host-pathogen interactions. This global approach, when combined with *in vivo* studies, can be an efficient strategy for identifying key factors involved in mycoplasma virulence and host-colonization, as well as a way to understand pathogenic processes involved in disease caused by these unconventional pathogens. The absence of small animal models for *M. agalactiae* and other ruminant mycoplasmal pathogens has been a significant bottleneck for functional genomics studies using large mutant libraries. High-throughput screening of more than 2000 individual clones by co-culture with host cells revealed 62 loci in the genome of *M. agalactiae* that were required for growth in this environment. The relevance of these loci in the biology of *M. agalactiae* when it replicates in its natural host remains to be determined, but experimental infections in lactating ewes have confirmed the essential role played by *nifS* in host-colonization (data not shown). Since the specific cell line was shown to influence which mutants were selected, comparative studies with different cell types, including differentiated monolayer cultures and cellular players involved in the immune response to infection, may provide further information about the interactions of mycoplasmas in these different cellular environments. Finally, these results provide an experimental framework for the development of control strategies, based on attenuated live vaccines, against mycoplasmosis in ruminant species.

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8. Supplementary data

Table S1. Degree of homology of CDS disrupted in *M. agalactiae* mutants with other ruminant mycoplasma species

CDS name [a]	Gene	Gene Product (COG) [b]	Predicted localization [c]	HGT [d]	Homolog in *M. bovis*	% identity / % similarity [e]	Homolog in *M. capricolum* subsp. *capricolum*	% identity / % similarity [e]
MAG0490		CHP	M		MBOVPG45_0056	64/85	-	0
MAG0640	*asnA*	Aspartate-ammonia ligase (E)	C	+	MBOVPG45_0071	88/94	MCAP0817	71/83
MAG0720	*nifS*	Cysteine desulfurase (E)	C		MBOVPG45_0081	79/89	MCAP0469	33/54
MAG0890	*hprK*	Hpr kinase phosphorylase (T)	C		MBOVPG45_0100	90/96	MCAP0777	33/52
MAG1180	*pepP*	XAA-PRO aminopeptidase (E)	C		MBOVPG45_0127	93/96	MCAP0341	38/61
MAG1330		CHP DUF285 family, predicted lipoprotein	M	+	MBOVPG45_0425	34/49	MCAP0300	40/50
MAG1430		HP	M		MBOVPG45_0157	73/84	-	0
MAG1490	*ldhD*	D lactate dehydrogenase (CHR)	IM	+	MBOVPG45_0163	94/95	-	0
MAG1500		Esterase lipase (R)	C	+	MBOVPG45_0164	83/89	MCAP0606	51/68
MAG1540	*tig*	Trigger factor (O)	C		MBOVPG45_0171	81/87	MCAP0517	29/50
MAG1740	*gidA*	Glucose inhibited division protein A (D)	C		MBOVPG45_0675	93/96	MCAP0856	48/66
MAG1860	*gidB*	Methyltransferase GidB (M)	C		MBOVPG45_0657	80/88	MCAP0807	34/50
MAG1890		HP	M		-	0	-	0
MAG2110		Protein phosphatase (T)	C		MBOVPG45_0630	85/91	MCAP0264	36/50
MAG2120	*pknB*	Serine/threonine-protein kinase (RTKL)	M		MBOVPG45_0629	81/88	MCAP0262	29/47
MAG2540		HP, Vpma like, predicted lipoprotein	M		-	0	-	0
MAG2680		HP	M		MBOVPG45_0550	64/71	-	0
MAG2870		CHP, predicted lipoprotein	M	+ x	MBOVPG45_0376	74/84	MCAP0350	40/59
MAG2930	*atpA*	ATP synthase α chain (C)	IM	+	MBOVPG45_0382	95/98	MCAP0358	74/87
MAG2960		CHP, predicted lipoprotein	M	+	MBOVPG45_0385	66/79	MCAP0072	45/63
MAG3030		HP	M		-	0	-	0
MAG3350		HP	M		-	0	-	0
MAG3370		CHP	M	+	MBOVPG45_0480	42/62	MCAP0568	36/56
MAG3480		HP	C		MBOVPG45_0439	56/71	-	0
MAG3720		CHP	IM		MBOVPG45_0461	70/83	-	0
MAG3740	*mraZ*	MraZ (S)	IM		MBOVPG45_0463	96/98	MCAP0387	28/57
MAG3790	*uvrA*	UvrABC system protein A (L)	C		MBOVPG45_0470	95/97	MCAP0774	56/73
MAG3860		CHP	IM	+	MBOVPG45_0479	96/97	MCAP0571	52/70
MAG4200		CHP	C		-	0	-	0
MAG4380		P115 like ABC transporter ATP binding protein (D)	C		MBOVPG45_0520	87/92	MCAP0495	44/65
MAG4650		Phosphomannomutase (G)	C		MBOVPG45_0350	82/92	MCAP0756	37/56
MAG4740		HP, predicted lipoprotein	M		MBOVPG45_0341	45/62	-	0
MAG4820		CHP (M)	C		MBOVPG45_0333	79/90	MCAP0866	25/46
MAG4950		HP, predicted lipoprotein	M	+	MBOVPG45_0320	73/85	MCAP0723	38/57
MAG5000		HP	M		MBOVPG45_0316	56/69	-	0
MAG5150		HP, predicted lipoprotein	M		MBOVPG45_0298	63/77	-	0
MAG5910		5' nucleotidase, predicted lipoprotein (F)	M		MBOVPG45_0690	88/94	-	0
MAG6090		HP, predicted lipoprotein	M		MBOVPG45_0710	42/63	-	0
MAG6450		CHP	C	+	MBOVPG45_0500	60/77	MCAP0494	61/73
MAG6690		HP	M	x	MBOVPG45_0767	82/88	-	0
MAG6760	*chrA*	Chromate transport protein (P)	M		MBOVPG45_0773	69/73	MCAP0198	33/46
MAG6770	*chrA*	Chromate transport protein (P)	M		MBOVPG45_0774	67/73	MCAP0197	41/60
MAG6870	*dnaX*	DNA polymerase III subunits gamma and tau (L)	C		MBOVPG45_0785	81/89	MCAP0008	42/60
MAG6960	*apt*	Adenine phosphorybosyltransferase (F)	C		MBOVPG45_0796	94/98	MCAP0497	46/66
MAG7100	*vpmaZ*	Variable surface lipoprotein D (VpmaZ precursor)	M		-	0	-	0
MAG7200	*scpB*	Segregation and condensation protein B (K)	C		MBOVPG45_0832	96/98	MCAP0601	26/52

[a] CDS found disrupted in *M. agalactiae* growth-deficient mutants [6].
[b] Hypothetical proteins (HP) have no homolog outside the *M. agalactiae* species. Conserved hypothetical proteins (CHP) share sequence similarity with proteins of unknown function identified in *Mollicutes* or other bacteria. COG categories of encoded proteins are indicated in parenthesis [38].
[c] Protein localization was predicted using TMHMM [40]; membrane (M), cytosolic (C), or indirectly linked to the membrane (IM).

[d] Genes supposed to have undergone horizontal gene transfer (HGT) between *M. agalactiae* and mycoplasmas from the mycoides cluster [6] are indicated by a plus sign (+). Genes displaying homologies with sequences in *M. gallisepticum* or *M. synoviae* genomes and supposed to have undergone HGT between these two species are identified by a cross sign (x) [6].
[e] Percentages of identity and similarity were determine by local BLAST using Molligen [42].

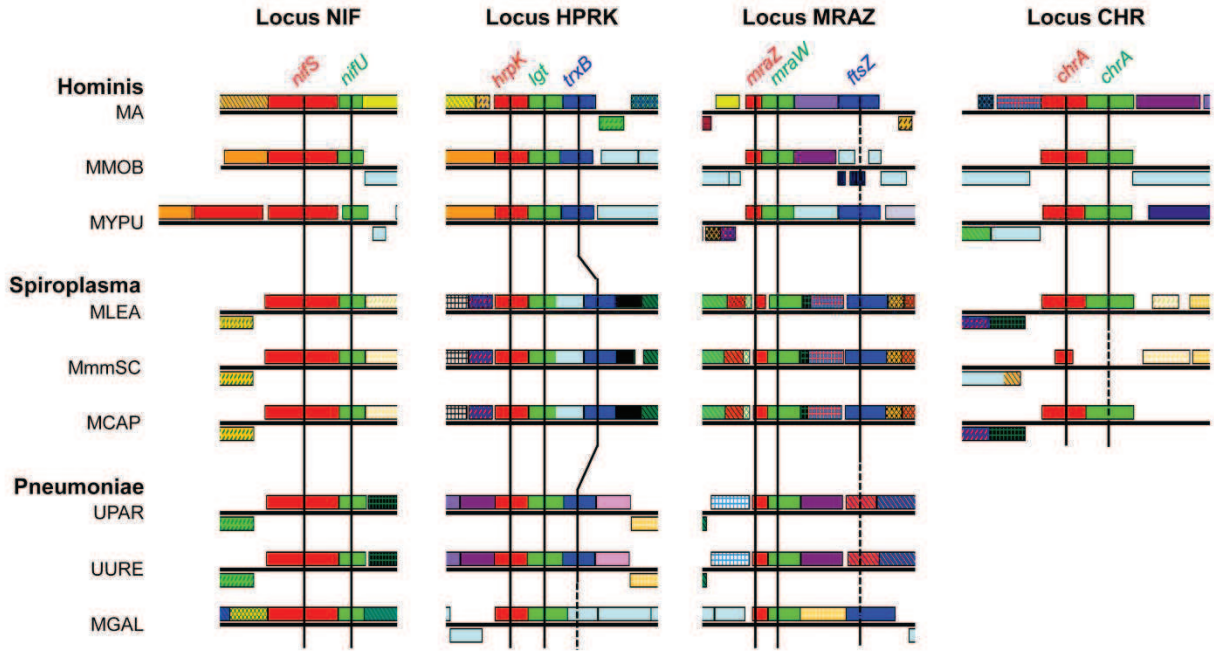


Figure S1. Genomic position of short CDS clusters NIF, HPRK, MRAZ and CHR in mycoplasma species with sequenced genomes. Gene organization of the conserved CDS clusters identified during the screening was analyzed using the Microbial Genome Database for Comparative Analysis software [40]. Homologues genes are indicated by the same color code and a black bar. Three examples were chosen for each phylogenetic group. In the case of the CHR locus, no homolog was identified in the Pneumoniae group. MA: *M. agalactiae*, MMOB: *M. mobile*, MYPU: *M. pulmonis*, MLEA : *M. leachii*, MmmSC : *Mycoplasma mycoides* subsp. *mycoides* SC, MCAP: *M. capricolum* subsp. *capricolum*, UPAR: *U. parvum*, UURE: *U. urealyticum*, MGAL: *M. gallisepticum*.

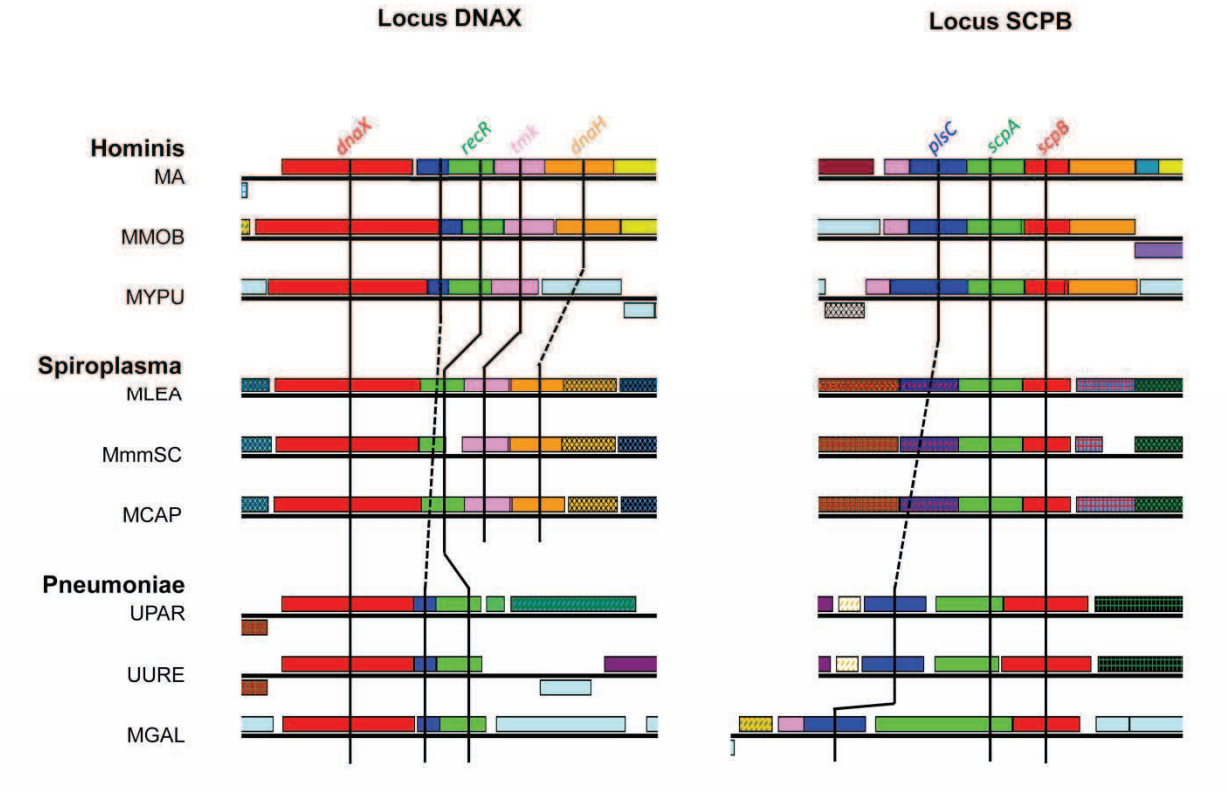


Figure S2. Genomic position of short CDS clusters PKNB and ATP in mycoplasma species with sequenced genomes. Gene organization of the conserved CDS clusters identified during the screening was analyzed using the Microbial Genome Database for Comparative Analysis software [40]. Homologues genes are indicated by the same color code and a black bar. Three examples were chosen for each phylogenetic group. MA: *M. agalactiae*, MMOB: *M. mobile*, MYPU: *M. pulmonis*, MLEA : *M. leachii*, MmmSC : *Mycoplasma mycoides* subsp. *mycoides* SC, MCAP: *M. capricolum* subsp. *capricolum*, UPAR: *U. parvum*, UURE: *U. urealyticum*, MGAL: *M. gallisepticum*.

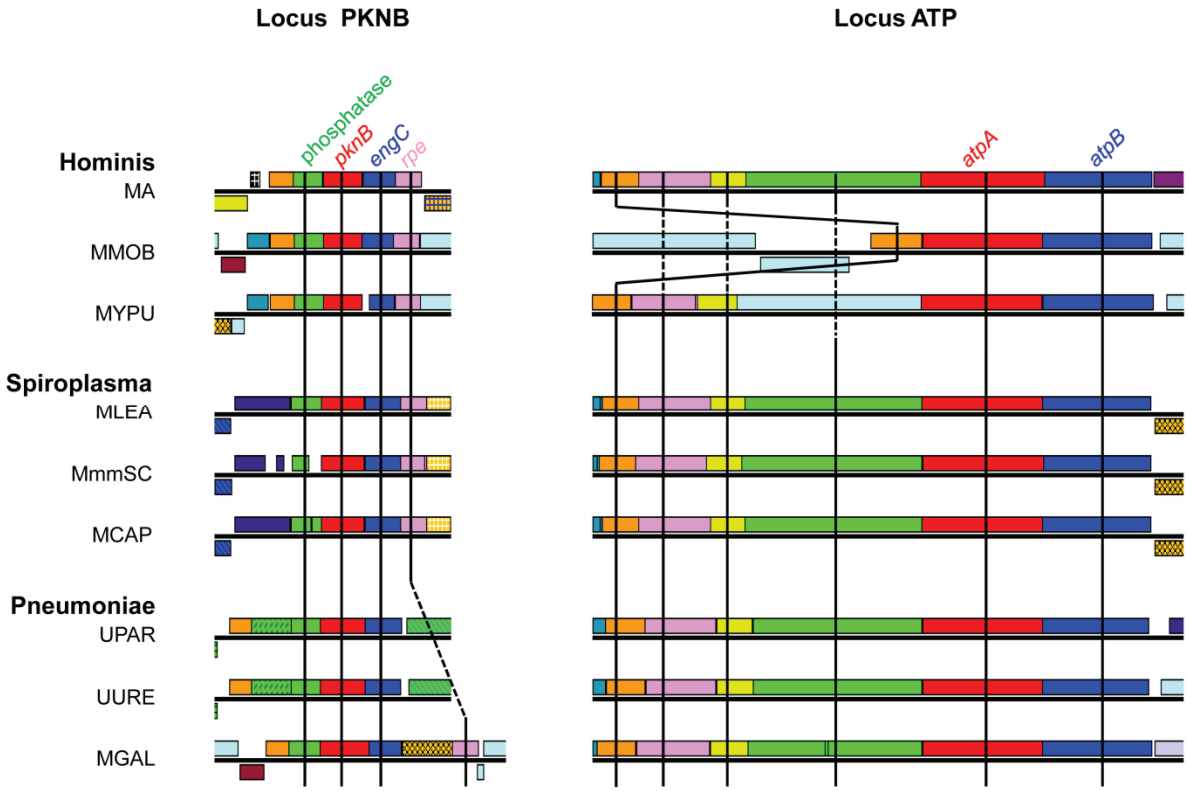


Figure S3. Genomic position of short CDS clusters DNAX and SCPB in mycoplasma species with sequenced genomes. Gene organization of the conserved CDS clusters identified during the screening was analyzed using the Microbial Genome Database for Comparative Analysis software [40]. Homologues genes are indicated by the same color code and a black bar. Three examples were chosen for each phylogenetic group. MA: *M. agalactiae*, MMOB: *M. mobile*, MYPU: *M. pulmonis*, MLEA : *M. leachii*, MmmSC : *Mycoplasma mycoides* subsp. *mycoides* SC, MCAP: *M. capricolum* subsp. *capricolum*, UPAR: *U. parvum*, UURE: *U. urealyticum*, MGAL: *M. gallisepticum*.

CHAPITRE 3

TROISIEME CHAPITRE : ANALYSE DES REGIONS INTERGENIQUES DE *MYCOPLASMA AGALACTIAE* IMPLIQUEES DANS L'INTERACTION AVEC LES CELLULES DE L'HOTE

1. Résumé

Mycoplasma agalactiae est l'agent étiologique de l'agalactie contagieuse et un modèle d'étude des mycoplasmoses de ruminants. Une précédente étude de génomique fonctionnelle, menée en culture cellulaire, suggère que certaines régions non codantes (NCRs) du génome de *M. agalactiae* pourraient être impliquées dans l'interaction de ce pathogène avec les cellules de l'hôte. Le présent travail a pour but l'analyse comparative des NCRs présentes dans le génome de *M. agalactiae* et de plusieurs espèces représentatives des différents groupes phylogénétiques de la classe des *Mollicutes*. Certaines des 9 NCRs identifiées chez *M. agalactiae* se distinguent par leur taille ou leur contenu en G+C. Aucune homologie avec des séquences connues d'ARNs non codants n'a pu être identifiée dans ces régions. Par contre, plusieurs NCRs contiennent des CDSs non identifiées lors de l'annotation du génome et codant pour des protéines de petite taille dont le rôle éventuel dans l'interaction de *M. agalactiae* avec les cellules de l'hôte reste à confirmer. D'autres NCRs sont des vestiges de CDSs. Des études de complémentation ont confirmé le rôle des NCRs dans l'interaction de *M. agalactiae* avec les cellules de l'hôte et suggèrent également un rôle potentiel des CDSs adjacentes. De manière générale, cette étude révèle la complexité des NCRs et propose des pistes pour mieux comprendre leur rôle dans les interactions hôte-mycoplasmes.

2. Introduction

La classe des *Mollicutes* rassemble l'ensemble des organismes communément appelés mycoplasmes. Elle est divisée en plusieurs genres parmi lesquels *Mycoplasma* et *Ureaplasma* regroupent la majorité des espèces identifiées à ce jour. Ces bactéries sont souvent décrites comme des organismes minimaux en raison de leurs génomes de petite taille (de 580 à 2200 kpb), de l'absence de paroi et de leurs capacités métaboliques limitées (Razin *et al.*, 1998). Malgré cette apparente simplicité, un certain nombre de mycoplasmes sont capables d'infecter l'homme et une grande variété d'espèces animales chez lesquelles ils provoquent des maladies à forte morbidité et à faible mortalité (Frey, 2002).

Parmi les espèces importantes en médecine vétérinaire, *Mycoplasma agalactiae* est l'agent étiologique à l'origine de l'agalactie contagieuse des petits ruminants. Cette mycoplasmose, inscrite sur la liste des maladies de l'Office International des Epizooties (OIE), se caractérise par des mammites, des arthrites et des kérato-conjonctivites (Bergonier *et al.*, 1997). Certaines espèces du groupe mycoides sont à l'origine de symptômes similaires chez les caprins. Il s'agit de *M. mycoides* subsp. *mycoides* Large Colony (LC), de *M. capricolum* subsp. *capricolum*. Des analyses *in silico* ont montré que *M. agalactiae* était phylogénétiquement distant du groupe mycoides mais que des transferts de gènes avaient eu lieu entre ces différentes espèces (Sirand-Pugnet *et al.*, 2007). A l'inverse, *M. agalactiae* est phylogénétiquement proche de *M. bovis* qui provoque des symptômes apparentés à ceux de l'agalactie contagieuse chez les grands ruminants (Pettersson *et al.*, 1996). Quelle que soit l'espèce considérée, les facteurs impliqués dans la virulence des mycoplasmes sont méconnus. Les avancées récentes dans le décryptage du génome de *M. agalactiae* (Sirand-Pugnet *et al.*, 2007; Nouvel *et al.*, 2010), le développement d'outils de génomique fonctionnelle (Baranowski *et al.*, 2010; Skapski *et al.*, 2011) et la possibilité de reproduire l'infection chez l'hôte naturel (MacOwan *et al.*, 1984; Hasso *et al.*, 1993; Buonavoglia *et al.*, 1999; Sanchis *et al.*, 1998, 2000) font de ce mycoplasme un modèle attractif pour l'étude du pouvoir pathogène des mycoplasmes de ruminants.

Chez les bactéries, l'étude des facteurs de virulence a longtemps été restreinte à l'analyse des régions codantes des génomes. Les régions intergéniques ou régions non codantes (en anglais Non Coding Region, NCR), par la présence de nombreux éléments régulateurs, peuvent également influencer le pouvoir pathogène de certaines espèces. Le contrôle de la transcription est l'exemple emblématique du rôle régulateur des NCRs. Chez les procaryotes, la plupart des données concernant la transcription ont été obtenues chez *Escherichia coli*. Les mécanismes mis en œuvre chez les mycoplasmes sont comparables, bien que quelques spécificités soient apparues. Les promoteurs de mycoplasmes ont une structure classique avec, en amont du site d'initiation de la transcription, une région -35 (séquence consensus

TTGACA) et une région -10 (séquence consensus TATAAT) séparées par 17 paires de bases (Razin *et al.*, 1998). Cependant, des études menées sur l'espèce *M. pneumoniae* ont montré que la région -35 était souvent moins conservée que la région -10 (Waldo *et al.*, 1999; Weiner *et al.*, 2000). Une autre particularité de la transcription chez les mycoplasmes est l'hétérogénéité de la taille des transcrits, qui résulte probablement d'une diminution des contraintes au niveau du site d'initiation, sans que l'on sache si cette hétérogénéité a un lien avec la régulation des gènes (Weiner *et al.*, 2000; Skapski *et al.*, 2011). La terminaison de la transcription semble être indépendante du facteur Rho car le gène codant pour celui-ci n'a pas été identifié chez les mycoplasmes. Par contre, des terminateurs de type Rho-indépendant, avec une tige et une boucle suivies d'un résidu poly U, ont été identifiés chez de nombreux mycoplasmes indiquant que ce type de terminaison est commun chez ces organismes (Muto et Ushida, 2002). Récemment, des études menées chez *M. genitalium* et *M. hyopneumoniae* ont mis en évidence l'existence de transcriptions dans les NCRs (Lluch-Senar *et al.*, 2007; Gardner et Minion, 2010). D'un point de vue physiologique, ces transcriptions semblent être une perte d'énergie, suggérant que les transcrits pourraient avoir un rôle important, comme la production de petits peptides ou d'ARNnc fonctionnels (Gardner et Minion, 2010).

L'identification récente de plus de 200 petits ARNs ou ARN non codants (ARNnc), d'une taille généralement inférieure à 500 nucléotides (Hüttenhofer et Vogel, 2006), dans les NCRs des génomes d'*Escherichia coli* mais aussi de *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Listeria monocytogenes* et de quelques espèces de cyanobactéries (Livny *et al.*, 2008), a suscité un regain d'intérêt pour les NCRs. Les ARNnc bactériens forment un groupe hétérogène de molécules impliquées dans la régulation des gènes, via la modulation de leur transcription et de leur traduction, et dans la stabilité des ARN messagers (ARNm) (Repoila et Darfeuille, 2008; Waters et Storz, 2009). Ils peuvent également jouer un rôle dans la virulence ou dans la réponse au stress (Gottesman, 2005; Geissmann *et al.*, 2006; Murphy et Payne, 2007; Padalon-Brauch *et al.*, 2008; Schiano *et al.*, 2010; Chabelskaya *et al.*, 2010; Podkaminski et Vogel, 2010). Les mécanismes d'action des ARNnc incluent (i) l'interaction avec des ARNm pour inhiber ou activer leur transcription, (ii) l'interaction avec des protéines régulatrices pour réprimer ou activer la transcription ou la traduction des cibles de ces protéines et (iii) la formation d'un complexe ARNnc-ARNm reconnu par la RNase III qui dégrade alors ce complexe (Geissmann *et al.*, 2006; Waters et Storz, 2009). Chez les *Mollicutes*, des petits ARNs ont été identifiés dans les génomes de *M. capricolum*, *M. pneumoniae* et *M. genitalium* sans qu'une fonction exacte ne puisse leur être attribuée (Ushida et Muto, 1993a, 1993b, 1995; Göhlmann *et al.*, 2000).

Comme les petits ARNs, les petites protéines ont longtemps été sous-estimées et fréquemment manqués lors de l'annotation des génomes. Des recherches récentes ont mis en évidence un nombre croissant de protéines d'une taille inférieure à 50 acides aminés lesquelles seraient impliquées dans de nombreux processus cellulaires. Certaines de ces protéines agissent comme des signaux et peuvent avoir (i) des rôles dans la communication intra-espèce, (ii) des effets inter-espèces, les antimicrobiens produits permettant de limiter la compétition dans une niche écologique donnée, ou (iii) un rôle dans les interactions hôte-pathogène. D'autres protéines de petites tailles peuvent agir comme des toxines, comme des chaperonnes présentant des substrats et des cofacteurs aux différents complexes enzymatiques de la cellule ou comme des stabilisateurs de ces mêmes complexes enzymatiques. Enfin, certaines jouent le rôle de régulateur en interagissant avec des protéines aux fonctions variées (Hobbs *et al.*, 2011). L'annotation des NCRs doit donc être considérée avec précaution. Par exemple, le petit ARN identifié chez *M. pneumoniae* et *M. genitalium* code en fait pour un petit peptide de 29 acides aminés dont la fonction demeure inconnue (Zimmerman et Herrmann, 2005).

Le rôle des NCRs chez les mycoplasmes demeure inexploré, mais des études récentes de génomique fonctionnelle menées au laboratoire suggèrent que certaines NCRs de *M. agalactiae* pourraient avoir une influence sur la croissance de ce mycoplasme en culture cellulaire (Baranowski *et al.*, 2010; Skapski *et al.*, 2011). En effet, le criblage d'une banque de mutants de *M. agalactiae*, construite par mutagenèse transpositionelle, a permis d'identifier 9 NCRs. L'interruption de ces NCRs, suite à l'insertion d'un transposon, est associée à une diminution des capacités de croissance de *M. agalactiae* en présence de cellules animales. Dans cette étude, nous avons analysé les caractéristiques des NCRs chez les *Mollicutes* et nous nous sommes intéressés aux particularités des 9 NCRs identifiées chez *M. agalactiae*. Cette étude a révélé la complexité de certaines NCRs et propose des pistes pour l'étude du rôle des NCRs dans l'interaction de *M. agalactiae* avec les cellules de l'hôte.

3. Matériels et méthodes

Souches bactériennes, lignées cellulaires et conditions de culture

La souche de référence PG2 de *M. agalactiae* (Sirand-Pugnet *et al.*, 2007) a été cultivée en milieu SP4 ou Aluotto, comme décrit précédemment (Baranowski *et al.*, 2010). Le mutant NCR B (mutant PG2 T10.260) (Tableau 1) a été isolé au cours de criblages d'une banque de mutants en culture cellulaire (Baranowski *et al.*, 2010; Skapski *et al.*, 2011) ; son génome se caractérise par la présence d'un transposon en position 388694. Le mutant NCR B a été cultivé en présence de gentamycine (50 µg/ml), ou de tétracycline (2 µg/ml) après transformation avec le plasmide p20-1 miniO/T et ses dérivés. Les titres des cultures de mycoplasmes ont été déterminés par des dilutions en série dans du DPBS (Dulbecco's Phosphate-Buffered Saline, Invitrogen) contenant 1 % de sérum de cheval décomplémenté (Invitrogen). Les dilutions (10 µl) ont été déposées sur gélose SP4 et les colonies de mycoplasmes dénombrées après 2 à 5 jours d'incubation à 37℃. La souche *Escherichia coli* DH10B (Invitrogen) a été utilisée pour le clonage et la propagation des plasmides. Les bactéries *E. coli* ont été cultivées en milieu Luria-Bertani (LB) en présence d'ampicilline (50 µg/ml) et/ou de tétracycline (2 µg/ml). La lignée cellulaire TIGMEC (T Immortalized Goat Milk Epithelial Cell), dérivée de cellules épithéliale caprines d'origine mammaire, a été décrite précédemment (Skapski *et al.*, 2011). Cette lignée cellulaire a été cultivée dans un milieu basé sur le DMEM (Dulbecco's Modified Eagle's Medium), composé de DMEM riche en glucose, additionné de pyruvate, de GlutaMAX-I (Invitrogen) et complémenté en acides aminés non essentiels (Invitrogen) et en sérum de veau fœtal décomplémenté (Eurobio) (Baranowski *et al.*, 2010).

Plasmides et constructions ADN

Le plasmide p20-1 miniO/T a été utilisé pour les études de complémentation des mutants de *M. agalactiae* (Baranowski *et al.*, 2010). Quatre constructions ont été générées pour complémenter le mutant NCR B. La région intergénique seule (position génomique 388097 à 388773) ou accompagnée d'un seul ou des deux gènes adjacents a été amplifiée par PCR à l'aide d'une polymérase à haute fidélité comme décrit précédemment (Skapski *et al.*, 2011). Les amorces ptsG_F1 (TCAAGCGTATC-CATGCTTGAGTCT) et MAG3260_R1 (AAAGCTGCAATAACAGGAAGCGA) (complémentation C1), ptsG_F1 et MAG3270_R1 (GCTTTATGCCTATATGCCTCTGCAA) (complémentation C2), MAG3240_F1 (GCATCAAAACATGGTCTAGGTACTGT) et MAG3260_R1 (complémentation C3) et MAG3240_F1 et MAG3270_R1 (complémentation C4) ont été utilisées (Tableau 1). Les produits PCR obtenus ont été clonés dans la plasmide pGEM-T Easy (Promega) puis dans le plasmide p20-1miniO/T comme décrit précédemment (Baranowski *et al.*, 2010). Les

constructions d'ADN ont été vérifiées par séquençage à l'aide des amorces M13-U (CGACGTTGTAAAACGACGGCCAGT), M13-30 (AACAATTTCACAC- AGGA-AACAGCTATGACC), MAG3260_R2 (ACCCCGTGTTTGGTTAGGTAATTGTTT) et MAG3260_R2 (TCAGCTCTTGAAAATGTGCGATACAA) ainsi que des amorces ptsG_F1, MAG3260_R1, MAG3240_F1 et MAG3270_R1.

Les transformations de *M. agalactiae* ont été réalisées par électroporation (Baranowski *et al.*, 2010). Après 3^H d'incubation en milieu non sélectif, les cellules ont été cultivées pendant 24^H en présence de l'antibiotique approprié puis étalées sur milieu gélosé sélectif. Les transformants ont été repiqués après 4 à 7 jours et cultivés en milieu SP4 sélectif.

Croissance de *M. agalactiae* en culture cellulaire

Les co-cultures ont été réalisées comme décrites précédemment (Baranowski *et al.*, 2010, Skapski *et al.,* 2011). Brièvement, les cellules TIGMEC ont été réparties dans des plaques de 24 puits (Falcon) à une densité de $2x10^4$ cellules/cm^2 et inoculées avec les différents mutants à $2x10^5$ CFU/ml. Après 3 jours, les co-cultures ont été soumis à un cycle de congélation/décongélation (-80℃/+37℃) et les myco plasmes ont été titrés.

Transcription inverse et amplification par PCR

L'extraction des ARN totaux par la méthode du TRIzol (Invitrogen) et les RT-PCR ont été réalisées dans les conditions décrites précédemment (Skapski *et al.*, 2011). Les oligonucleotides SG5 (TTTTACACAATTATACGGACTTTATC) et ptsG_F1 ont été utilisées comme amorces.

Analyses de séquences *in silico*

Le logiciel Artemis (Rutherford *et al.*, 2000) a été utilisé pour l'analyse des séquences intergéniques. L'ensemble des programmes Blast (http://www.ncbi.nlm.nih.gov/BLAST/) ont été utilisés pour la recherche d'homologies de séquences. Les recherches de petits ARN non codants dans le génome de la souche PG2 ont été réalisées à l'aide de l'outil RNAspace (http://rnaspace.org/).

4. Résultats et Discussion

Analyse des NCRs au sein des *Mollicutes*

Afin de mieux comprendre l'organisation des NCRs chez les mycoplasmes, une analyse comparative a été réalisée sur onze génomes représentatifs des principaux groupes phylogénétiques qui composent la classe des *Mollicutes* (Tableau 1). Six génomes ont été sélectionnés dans le groupe phylogénétique *Hominis*, avec deux souches pour *M. agalactiae* (PG2 et 5632) et *M. bovis* (PG45 et Hubei), et une souche pour les espèces *M. hominis* et *M. pulmonis*. Nous avons élargi notre sélection au groupe *Pneumoniae* avec les espèces *M. genitalium* et *M. gallisepticum*, au groupe *Spiroplasma* avec les mycoplasmes de ruminants *M. mycoides* subsp. *mycoides* SC (MmmSC) et *M. mycoides* subsp. *mycoides* LC (MmmLC), et finalement au groupe *Phytoplasma/Acholeplasma* avec *Acholeplasma laidlawii*. Les NCRs de ces différents génomes ont été dénombrées et leur contenu en G+C calculé (Tableau 1). La densité des NCRs varie entre 8,2 % et 12,7 %, avec une valeur intermédiaire de 10,1 % chez *M. agalactiae*. Ces différences ne semblent dépendre ni de la taille du génome, ni du groupe phylogénétique, puisque MmmSC et MmmLC, deux espèces du groupe *Spiroplasma* dont la taille des génomes est comparable, contiennent respectivement 12,7 % et 8,6 % de NCRs. La densité des NCRs chez les *Mollicutes* est légèrement plus faible que chez des bactéries « classiques » comme *Clostridium perfringens* (15,6 %) ou *Escherichia coli* (13,0 %), et reflète la forte densité en CDSs des génomes de mycoplasmes (Razin *et al.*, 1998). La distribution des bases G et C dans les génomes des mycoplasmes est hétérogène (Razin *et al.*, 1998), avec pour les NCRs un contenu en G+C inférieur à celui du génome total (Tableau 1).

Tableau 1. Comparaison des génomes de *Mollicutes*

Espèce (souche)	Groupe phylogénétique	Taille du génome (bp)	% G+C total	Nombre NCRs [a]	% NCRs	% G+C NCRs
M. agalactiae (PG2)	*Hominis*	877438	29,70	659	10,1	23,95
M. agalactiae (5632)	*Hominis*	1006702	29,62	731	10,1	23,56
M. bovis (Hubei)	*Hominis*	948121	29,29	695	9,7	22,62
M. bovis (PG45)	*Hominis*	1003404	29,31	737	10,0	23,12
M. pulmonis (UAB CTIP)	*Hominis*	963879	26,64	617	9,3	18,54
M. hominis (PG21)	*Hominis*	665445	27,12	446	10,2	20,92
M. mycoides subsp. *mycoides* SC (PG1)	*Spiroplasma*	1211703	23,97	891	12,7	21,88
M. mycoides subsp. *mycoides* LC (95010)	*Spiroplasma*	1153998	23,82	835	8,6	19,35
M. genitalium (G37)	*Pneumoniae*	580076	31,69	325	8,2	30,52
M. gallisepticum (Rlow)	*Pneumoniae*	1012800	31,47	636	10,9	25,24
A. laidlawii (PG8A)	*Phytoplasma Acholeplasma*	1496992	31,93	1090	9,3	28,45

[a] Régions non codantes (en anglais, Non Coding Regions : NCRs)

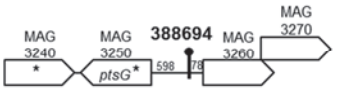
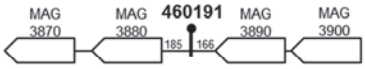
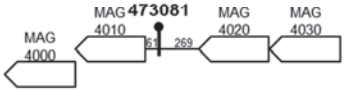
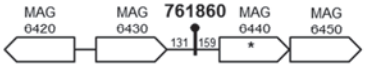
Analyse des NCRs de *M. agalactiae* identifiées par criblage en culture cellulaire

Les 9 NCRs identifiées ont été désignées NCR A à NCR I (Tableau 2), dans leur ordre d'apparition sur le génome de la souche de référence PG2 (Skapski *et al.*, 2011). Ces NCRs ont été classées en 4 types selon l'orientation des gènes adjacents (Tableau 2 et Fig. 1A). Les types I et II correspondent aux NCRs encadrées respectivement par deux CDSs de même orientation, positive ou négative par rapport au génome. Les NCRs des types III et IV sont encadrées par des CDSs en orientation inverse.

Une analyse des génomes de *M. agalactiae* (souches PG2 et 5632) et de *M. bovis* (PG45 et Hubei) montre que les différents types de NCRs sont en proportion comparables dans ces génomes, avec une large majorité de NCRs encadrées par des gènes de même orientation (type I ou II) (Fig. 1A et 1B). Les 9 NCRs identifiées lors du criblage en culture cellulaire respectent ces proportions (Tableau 2 et Fig. 1B). La taille des NCRs de type III des génomes de PG2, 5632, Hubei et PG45 est en moyenne 2 à 3 fois plus grande que la taille des autres NCRs (Fig. 2A), et les NCRs de type IV sont en moyenne 1,1 à 1,3 fois plus riches en G+C (Fig. 2B). La présence de terminateurs riches en G+C dans les régions de type IV pourrait être à l'origine de cette différence.

Certaines des NCRs sélectionnées lors du criblage de *M. agalactiae* PG2 en culture cellulaire se distinguent par une taille et un contenu en G+C différents des valeurs moyennes observées pour les NCRs de la souche PG2 (Tableaux 1 et 2). Ainsi, parmi les 9 NCRs identifiées, 6 possèdent une taille supérieure à 300 nucléotides (NCRs B, C, D, E, F et G ; Tableau 2 et Fig. 3A), alors que 85 % des NCRs du génome de PG2 ont une taille inférieure à cette valeur (Fig. 3A). Un pourcentage similaire a été observé pour la souche 5632 de *M. agalactiae* et les deux souches de *M. bovis* analysées. L'origine de cette différence n'est pas connue, mais la probabilité d'observer un évènement d'insertion lors de la mutagénèse transpositionelle augmente avec la taille de la région. De même, 5 des 9 NCRs identifiées lors du criblage ont un contenu en G+C supérieur à 25 % (NCRs A, D, E, F et H ; Tableau 2 et Fig. 3B), alors que 65 % des NCRs du génome de PG2, 5632, Hubei et PG45 ont un contenu en G+C inférieur à 25 % (Fig. 3B). Le contenu en G+C des NCRs A, D, E, F et H est donc plus proche de celui des CDSs. Ces NCRs pourraient donc contenir des CDSs de petite taille non annotées ou dériver de CDSs dégénérées dépourvues de codon START. La présence de terminateurs riches en G+C pourrait également être à l'origine de ces différences pour les NCRs de petite taille.

Tableau 2. NCRs de *M. agalactiae* identifiées par criblage de mutants en culture cellulaire [a]

Nom [b]	Taille (nt)	Cellules [c]	Nb de mutants (Nb de sites d'insertion) [d]	% G+C [e]	Type [f]	Nb d'ORF identifiées [g]	Environnement [h]
NCR A	67	F	1 (1)	36,36	I	0	
NCR B	676	E, F, H	7 (1)	18,96	III	2	
NCR C	740	E, F, H	2 (1)	23,27	II	4	
NCR D	351	F	1 (1)	29,71	II	3	
NCR E	423	F, H	1 (1)	26,30	IV	2	
NCR F	330	F, H	2 (1)	25,84	II	2	
NCR G	802	E	1 (1)	22,35	II	5	
NCR H	290	F	1 (1)	27,34	I	0	
NCR I	80	F, H	2 (2)	22,78	II	0	

[a] D'après Baranowski *et al.*, 2010 ; Skapski *et al.*, 2011

[b] Les NCRs ont été désignées par une lettre selon leur position dans le génome de *M. agalactiae* (Skapski *et al.*, 2011). Leur position est indiquée en gras dans la colonne environnement.

[c] Les lettres E et F désignent respectivement les NCRs identifiées sur les cellules épithéliales TIGMEC et sur les cellules fibroblastiques TIGEF. La lettre H indique les NCRs qui ont également été identifiées lors d'un criblage sur cellules HeLa.

[d] Pour chaque région, le nombre de mutants identifiés au cours du criblage sur cellules caprines est indiqué ainsi que le nombre de sites d'insertion différents.

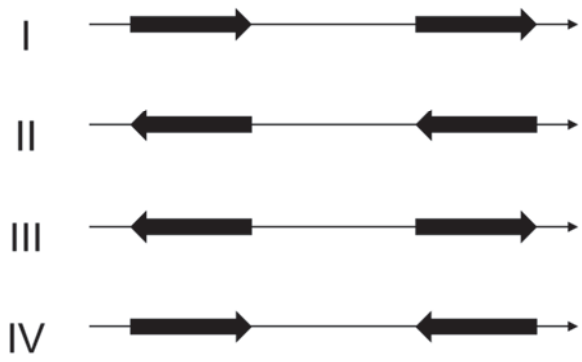
[e] Pour chaque NCR, le % en G+C a été calculé à l'aide du logiciel ARTEMIS (Rutherford *et al.*, 2000).

[f] Le type de NCR dépend de l'orientation des gènes adjacents à chaque région. Le type I et II correspondent aux NCRs encadrées respectivement par deux CDSs de même orientation, positive ou négative par rapport au génome. Les NCRs des types III et IV sont délimitées par des CDSs en orientation inverse.

[g] Pour chaque région, le nombre d'ORF d'une taille inférieure à 50 acides aminés identifiées à l'aide du logiciel ARTEMIS est indiqué. Les ORFs de plus de 50 acides aminés situées à cheval sur une région intergénique et une région codante ne sont pas identifiées dans cette étude lorsque la partie située dans la région intergénique est inférieure à 50 acides aminés.

[h] Pour chaque région, les sites d'insertion des transposons ont été déterminés par séquençage direct de l'ADN génomique et leur position, indiquée en gras, a été définie en se basant sur la séquence publiée (NC_009497). Les gènes sont identifiés par leur mnémonique ainsi que par leur nom, pour les gènes ayant une fonction attribuée. Les CDS identifiées au cours d'une analyse protéomique sont indiquées par un astérisque (*) (Nouvel *et al.*, 2010). Les CDS identifiées à l'aide de l'outil AMIGène (Bocs *et al.*, 2003) et non annotées dans le génome de PG2 sont représentées par des flèche noires.

A



B

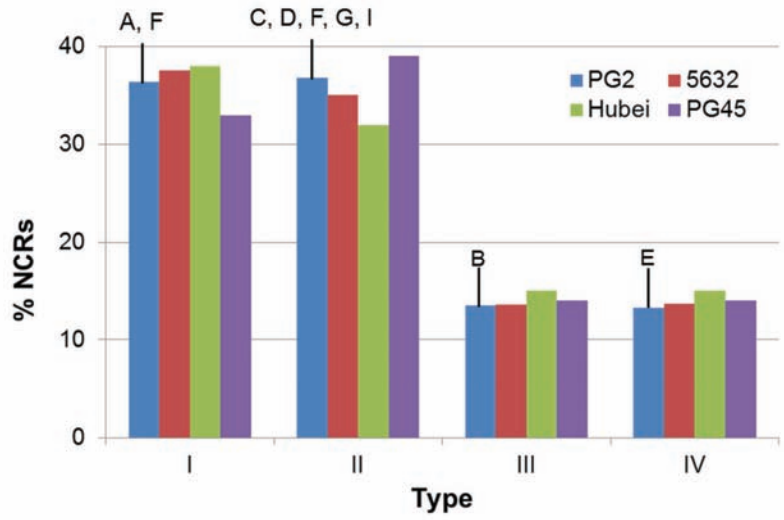
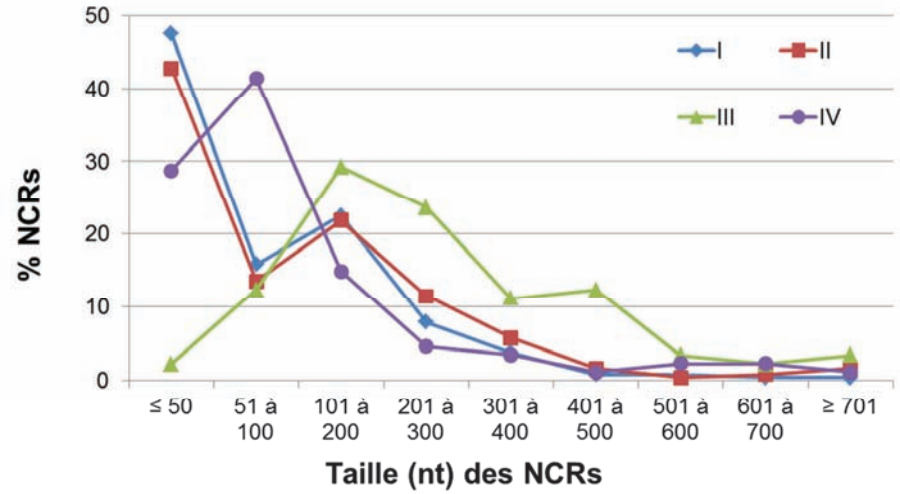


Figure 1. Organisation des NCRs dans les génomes de PG2, 5632, PG45 et Hubei. (A) L'orientation des gènes qui encadrent les NCRs permet de définir quatre types de région. Les types I et II correspondent aux NCRs encadrées respectivement par deux CDSs de même orientation, positive ou négative par rapport au génome. Les NCRs de type III et IV sont délimitées par des CDSs en orientation opposée. Compte tenu de leur configuration, les régions de type III sont plus susceptibles de contenir des régions promotrices et les régions de type IV des terminateurs. (B) Proportion des différents types de NCRs dans les génomes des souches *M. agalactiae* PG2 et 5632 et des souches *M. bovis* Hubei et PG45. Les 9 NCRs sélectionnées lors du criblage en culture cellulaire sont identifiées par une lettre (Tableau 2).

A



B

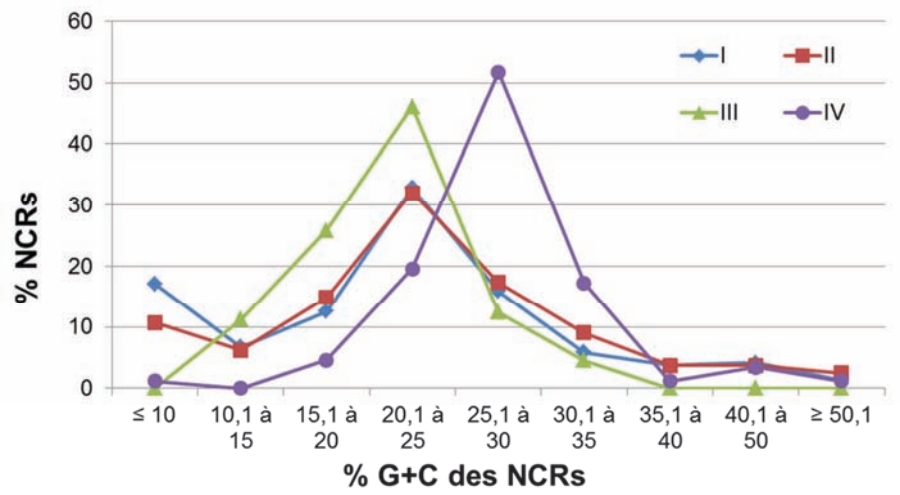
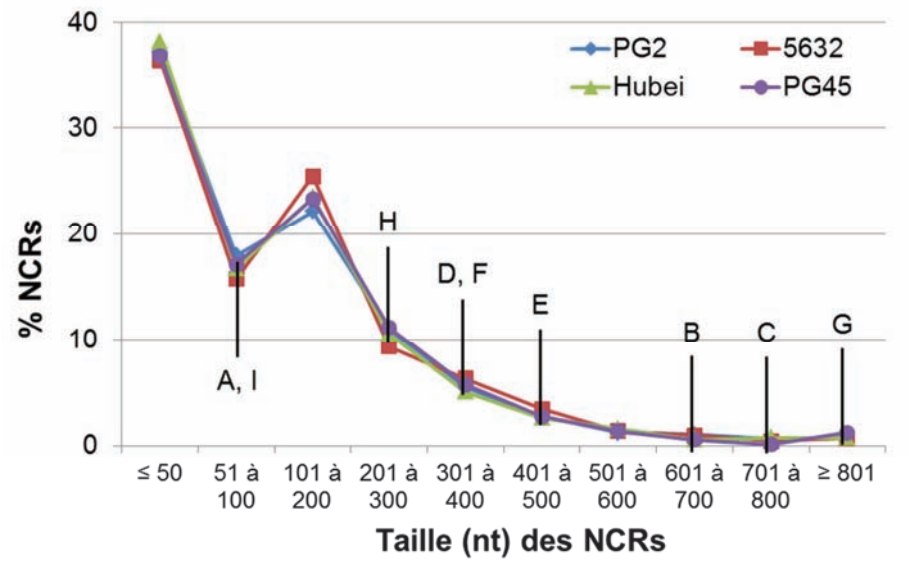


Figure 2. Analyse des NCRs dans le génome *M. agalactiae*. Proportion des NCRs de la souche PG2 en fonction de leur taille (A) et de leur contenu en G+C (B). Les NCRs de type I et II correspondent aux régions encadrées respectivement par deux CDSs de même orientation, positive ou négative par rapport au génome. Les NCRs de type III et IV sont délimitées par des CDSs en orientation opposée (Figure 1).

A



B

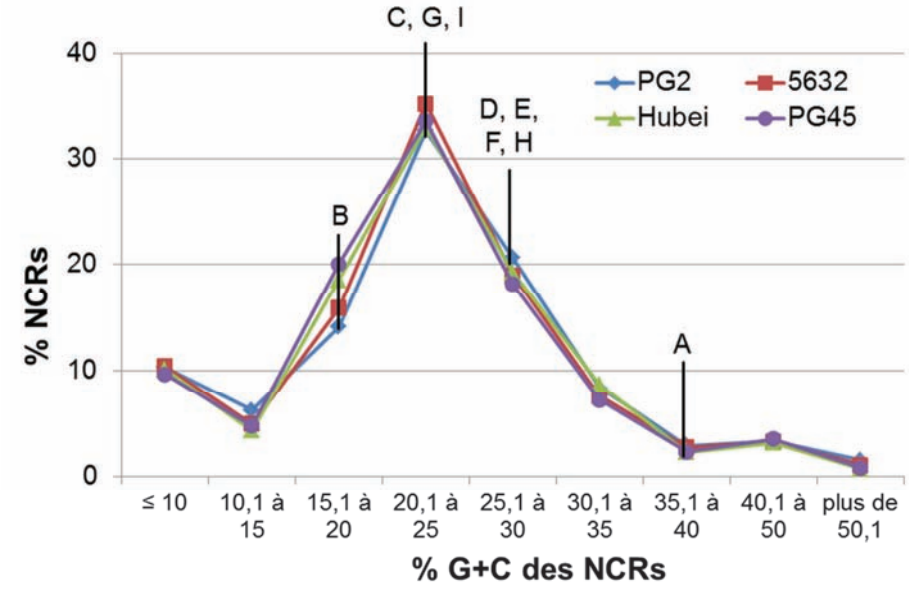


Figure 3. Analyse des NCRs des génomes de PG2, 5632, PG45 et Hubei. Proportion des NCRs dans le génome des souches PG2 et 5632 de *M. agalactiae* et dans le génome des souches PG45 et Hubei de *M. bovis* en fonction de leur taille (A) et de leur contenu en G+C (B). Les 9 NCRs sélectionnées lors du criblage en culture cellulaire sont identifiées par une lettre (Tableau 2).

Recherche de CDSs codant pour des peptides de petite taille dans les NCRs

La découverte d'une CDS (séquences en acides aminés entre un codon START et un codant STOP) codant pour un peptide de 29 acides aminés chez les espèces *M. pneumoniae* et *M. genitalium* (Zimmerman et Herrmann, 2005) indique que certaines des 9 NCRs de *M. agalactiae* identifiées en culture cellulaire pourraient contenir des CDSs de petite taille qui n'auraient pas été retenues lors de l'annotation du génome de PG2. La recherche d'ORFs (séquences en acides aminés entre deux codons STOP) sur l'ensemble des NCRs de la souche type PG2 a permis d'identifier 214 ORFs d'une taille supérieure à 50 acides aminés. Ces ORFs pourraient coder pour des peptides de 50 à 178 acides aminés. Parmi celles-ci, 18 ont été identifiées dans les 9 NCRs sélectionnées lors du criblage, dont 3 CDSs de petite taille détectées dans les régions NCRs C, E et G, grâce à l'utilisation de l'outil d'annotation AMIGene. Ces CDSs codent respectivement pour des protéines de 69, 52 et 67 acides aminés. L'expression de ces trois petites protéines n'a pas pu être vérifiée par l'analyse du protéome total des souches PG2 et 5632 (Nouvel *et al.*, 2010). Toutefois, des contraintes techniques limitent la détection aux protéines de taille supérieure à 100 acides aminés environ. L'expression et le rôle éventuel de ces protéines de petite taille dans l'interaction de *M. agalactiae* avec les cellules de l'hôte restent à démontrer, mais l'interruption des CDSs identifiées dans les régions NCRs E et G (Tableau 2) rend cette hypothèse séduisante.

Conservation des NCRs chez *M. agalactiae* et *M. bovis*

Les 9 NCRs identifiées chez *M. agalactiae* montrent des homologies de séquence avec les génomes des souches *M. agalactiae* 5632 et/ou *M. bovis* PG45 (Tableau 3).

La NCR I et les gènes adjacents sont parfaitement conservés chez 5632 et PG45 (Fig. 4A). Les NCRs G et H de PG2 sont parfaitement conservées mais uniquement chez 5632 (Fig. 4A). Inversement, les NCRs D, E et F ne présentent pas d'homologies de séquences avec 5632, mais sont conservées chez PG45 (Fig. 4B). Chez PG2, ces régions sont situées dans un locus de 20 kpb codant pour un vestige d'ICE (Sirand-Pugnet *et al.*, 2007; Nouvel *et al.*, 2010). La NCR E est dupliquée chez PG45 et la CDS de petite taille identifiée dans cette NCR est conservée chez PG45 (MBOVPG45_0205 et MBOVPG45_0486). Les séquences homologues des NCRs D et F chez PG45 font partie des CDSs MBOVPG45_0481 et MBOVPG45_0492 située dans l'une des deux ICEs de *M. bovis*. Ces résultats suggèrent que la NCR D chez PG2, ainsi que les CDS adjacentes MAG3880 et MAG3890, pourraient avoir évolué à partir d'une CDS unique homologue à MBOVPG45_0481. Les CDS adjacentes seraient alors des pseudogènes de cette CDS unique, tronqués en N- et C-terminal. De manière similaire, la NCR F de PG2 et la CDS adjacente MAG4010 pourrait avoir évolué à

partir d'une CDSs unique, homologue à MBOVPG45_0492. Les NCRs D et F se distinguent par un contenu en C+G élevé, proche du contenu en G+C des CDSs.

La séquence homologue de la NCR A chez 5632 et chez PG45 fait partie des CDSs MAGa0320 et MBOVPG45_0031 (Fig. 4B). De la même façon que pour la NCR D, la NCR A chez PG2 ainsi que les CDSs MAG0310 et 0320 adjacentes pourraient avoir évolué à partir d'une CDS unique, homologue à la CDS MAGa0320 ou MBOVPG45_0031. La NCRs A se distingue également par un contenu en C+G élevé.

Les régions NCR B et NCR C font partie de régions génomiques très variables entre les souches PG2 et 5632, avec des réarrangements complexes de l'ADN (Nouvel *et al.*, 2010). Seule l'extrémité 3' de la NCR B est conservée chez 5632 et PG45, en amont d'un gène transféré entre *M. agalactiae* et les mycoplasmes du groupe mycoides. Cette extrémité 3' présente également une homologie partielle avec une région intergénique (région génomique 691943 à 692084) de *M. capricolum* subsp. *capricolum* (nucléotides 511 à 656 de NCR B, 69 % d'identité). Cette dernière région est hautement conservée au sein du groupe mycoides (*M. mycoides* subsp. *mycoides* SC : 96 % d'identité ; *M. leachii* : 95 % d'identité). Cette partie de la NCR B pourrait donc avoir été transférée en même temps qu'un gène *drp,* caractérisé par la présence d'un motif DUF285 (voir Analyse de la NCR B). En ce qui concerne la région C, seule la partie 5' est présente dans la souche 5632 suite au réarrangement, ce qui explique l'homologie locale détectée sur cette partie de la séquence uniquement.

Les NCRs isolées au cours du criblage en culture cellulaire sont, pour la plupart, encadrées par des protéines hypothétiques ou des protéines hypothétiques conservées. La majorité de ces protéines n'ont pas été identifiées au cours d'une analyse de protéomique réalisée en milieu axénique (Tableau 2) (Nouvel *et al.*, 2010). Pour les régions A, B, E, F, G et I, l'insertion du transposon pourrait potentiellement altérer un promoteur, un terminateur ou interrompre une CDS codant un peptide de petite taille. Dans le cas des régions C, D et H, l'effet de l'insertion du transposon reste plus difficile à expliquer. La complexité de chacune de NCRs identifiées montre la nécessité de les étudier chacune spécifiquement et dans le détail pour comprendre le phénotype observé en culture cellulaire.

Tableau 3. Conservation des NCRs de *M. agalactiae* identifiées par criblage en culture cellulaire [a]

Régions intergéniques	Taille (nt)	*M. agalactiae* 5632		*M. bovis* PG45 [b]	
		Identité (%)	Région [c]	Identité (%)	Région [c]
NCR A	67	93	1 - 67	83	16 - 62
NCR B	676	90	295 - 676	94	288 - 676
NCR C	740	93	1 - 411	aucune	aucune
NCR D	351	aucune [d]	aucune	83	6 - 351
NCR E	432	aucune	aucune	96	1 - 423
NCR F	330	aucune	aucune	95	1 - 330
NCR G	802	90	1 - 802	aucune	aucune
NCR H	290	89	1 - 290	aucune	aucune
NCR I	80	87	1 - 71	83	2 - 77

[a] D'après Baranowski *et al.*, 2010 ; Skapski *et al.*, 2011.
[b] les résultats obtenus sont identiques pour les souches *M. bovis* PG45 et Hubei.
[c] la partie de la NCR sur laquelle le pourcentage d'identité a été calculé est indiquée en nucléotides.
[d] Absence d'homologie.

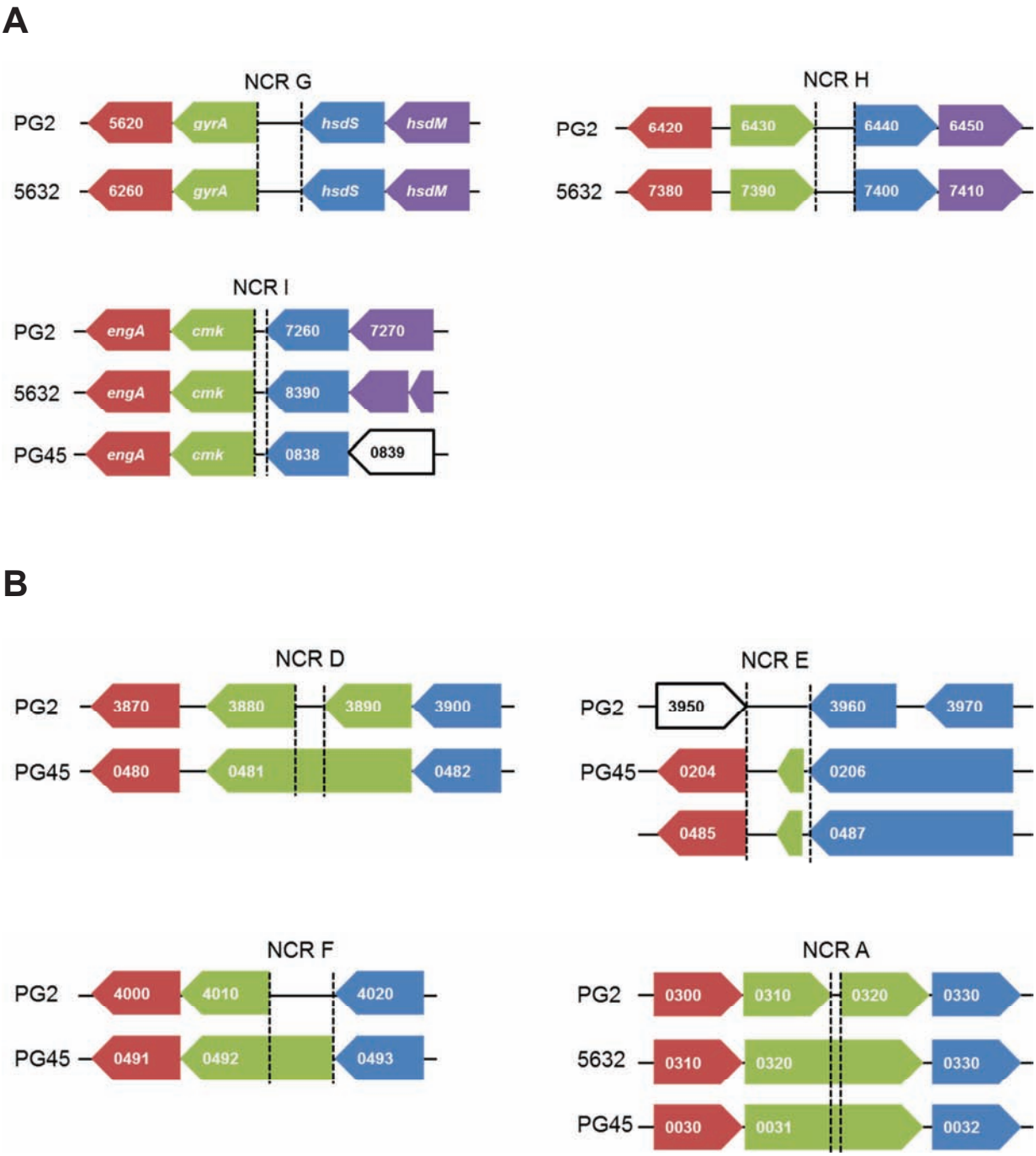


Figure 4. Représentation schématique des NCRs de *M. agalactiae* identifiées par criblage en culture cellulaire. Les NCRs identifiées dans le génome de la souche PG2 et les CDSs adjacentes sont représentées schématiquement et comparées aux régions homologues dans le génome de la souche 5632 de *M. agalactiae* ou PG45 de *M. bovis*. Les CDSs homologues sont indiquées par un même code de couleur. (A) Régions hautement conservées entre PG2 et 5632 et/ou entre PG2 et PG45. (B) Régions faiblement conservées entre PG2 et 5632 et/ou entre PG2 et PG45. Les NCRs A, D et F sont probablement des vestiges de CDSs. La NCR E est dupliquée chez PG45 et encode une protéine de petite taille.

Analyse de la NCR B

La NCR B a été identifiée à plusieurs reprises lors du criblage en culture cellulaire (Baranowski *et al.*, 2010 ; Skapski *et al.*, 2011). Les mutants NCR B présentent une réduction de leurs capacités réplicatives en cultures cellulaire qui se manifeste, avec les cellules TIGMEC, par une diminution de leur titre final de plus de 87 % (0,9 logarithme) par rapport à la souche parentale (Fig. 5A).

Chez les mutants NCR B identifiés, le transposon est inséré 78 nucléotides en amont du gène MAG3260 (Tableau 1) qui appartient à la famille *drp*. Les gènes *drp* ont été transférés entre *M. agalactiae* et les mycoplasmes du groupe mycoides et codent pour des protéines hypothétiques contenant un ou plusieurs domaines DUF285. Le domaine DUF285 a été identifié chez d'autres bactéries mais pas chez les *Mollicutes*, à l'exception du cluster mycoides (Sirand-Pugnet *et al.*, 2007). Une analyse récente de protéomique a montré que le produit de certains gènes *drp* était présent dans la fraction membranaire, suggérant que cette famille de gènes participe à la diversité de surface des mycoplasmes (Nouvel *et al.*, 2010). A l'extrémité 5' de la région intergénique se trouve le gène *ptsG* (Tableau 1) qui code pour la sous-unité apportant la spécificité pour le glucose au système PTS (système de phosphotransférase dépendant du phosphoénolpyruvate). La souche PG2 possède deux copies du gène *ptsG* (Nouvel *et al.*, 2010). Ces deux gènes codent pour des protéines de 123 acides aminés qui présentent 81 % d'identité.

Afin de confirmer le rôle de la NCR B dans la croissance de *M. agalactiae* en culture cellulaire, des tests de complémentation du mutant NCR B ont été réalisés à l'aide de différentes constructions plasmidiques (Fig. 5B). Dans la construction C1, la séquence correspondant à la NCR B de PG2 a été clonée dans le vecteur p20.1 mini O/T. Les constructions C2 et C3, incluent la séquence NCR B accompagnée respectivement du gène adjacent MAG3260 ou *ptsG*. Enfin, la construction 4 inclut la séquence NCR B accompagnée des deux gènes adjacents. Le mutant NCR B a été transformé par les constructions C1, C3 et C4, mais des tentatives répétées de transformation avec la construction C2 ont échoué, suggérant que cette construction était probablement létale. Un résultat identique a été obtenu lors d'un essai de transformation de la souche parentale PG2. Enfin, la souche PG2 et le mutant NCR B ont été transformés avec le vecteur vide p20.1 mini O/T afin de générer les transformants PG2-O/T et NCR B-O/T.

En milieu axénique, la NCR B n'a aucune influence sur la croissance de *M. agalactiae* et le mutant NCR B présente une croissance identique à la souche PG2 (Fig. 5C). De la même manière, la croissance des transformants NCR B-O/T et PG2-O/T ne montrent pas de différence, bien que leur croissance en présence de tétracycline soit retardée par rapport aux

souches parentales (Fig. 5C). Le coût infligé par le maintien du marqueur de sélection en conditions axéniques est identique pour tous les transformants testés.

Les croissances en culture cellulaire ont été réalisées sur cellules TIGMEC, en absence de tétracycline (Fig. 5D). Après co-culture, les mycoplasmes transformants ont été dénombrés en double, en présence et en absence de tétracycline. Les résultats des titrages montrent une perte rapide du marqueur de sélection en absence de pression de sélection. Toutefois, les titrages réalisés en présence de tétracycline indiquent que la construction plasmidique C4 restaure une croissance du mutant NCR B à un niveau comparable à celui du transformant PG2-O/T (Fig. 5D). Une réduction de 66 % du titre final a été observée pour le transformant NCR B-O/T par rapport à PG2-O/T. Les constructions plasmidiques C1 et C3 ont permis respectivement de faire chuter ce pourcentage à 42 % et 27 % (Fig. 5D). La complémentation du mutant NCR B semble confirmer le rôle de cette région dans l'interaction de *M. agalactiae* avec les cellules de l'hôte. Toutefois, les CDSs adjacentes (*ptsG* et MAG3260) pourraient également être impliquées dans cette interaction, puisque la construction plasmidique C1, contenant uniquement la séquence de la NCR, ne restaure pas complètement la croissance du mutant NCR B. L'identification de régions promotrices dans la séquence du transposon utilisé pour la mutagénèse (Skapski *et al.*, 2011) suggère qu'une initiation de la transcription à partir du transposon inséré dans la NCR B pourrait modifier le niveau d'expression des gènes adjacents. Cependant, des essais d'amplifications par RT-PCR n'ont pas permis de vérifier cette hypothèse (données non présentées).

Recherche d'ARNnc dans le génome de *M. agalactiae*

L'analyse du génome de la souche PG2 à l'aide de l'outil RNAspace n'a révélé aucune séquence homologue aux ARNnc décrits chez d'autres bactéries. L'évolution rapide des génomes de mycoplasmes pourrait être à l'origine de ce résultat. Une approche expérimentale pourrait permettre d'identifier ce type de molécules chez ces organismes.

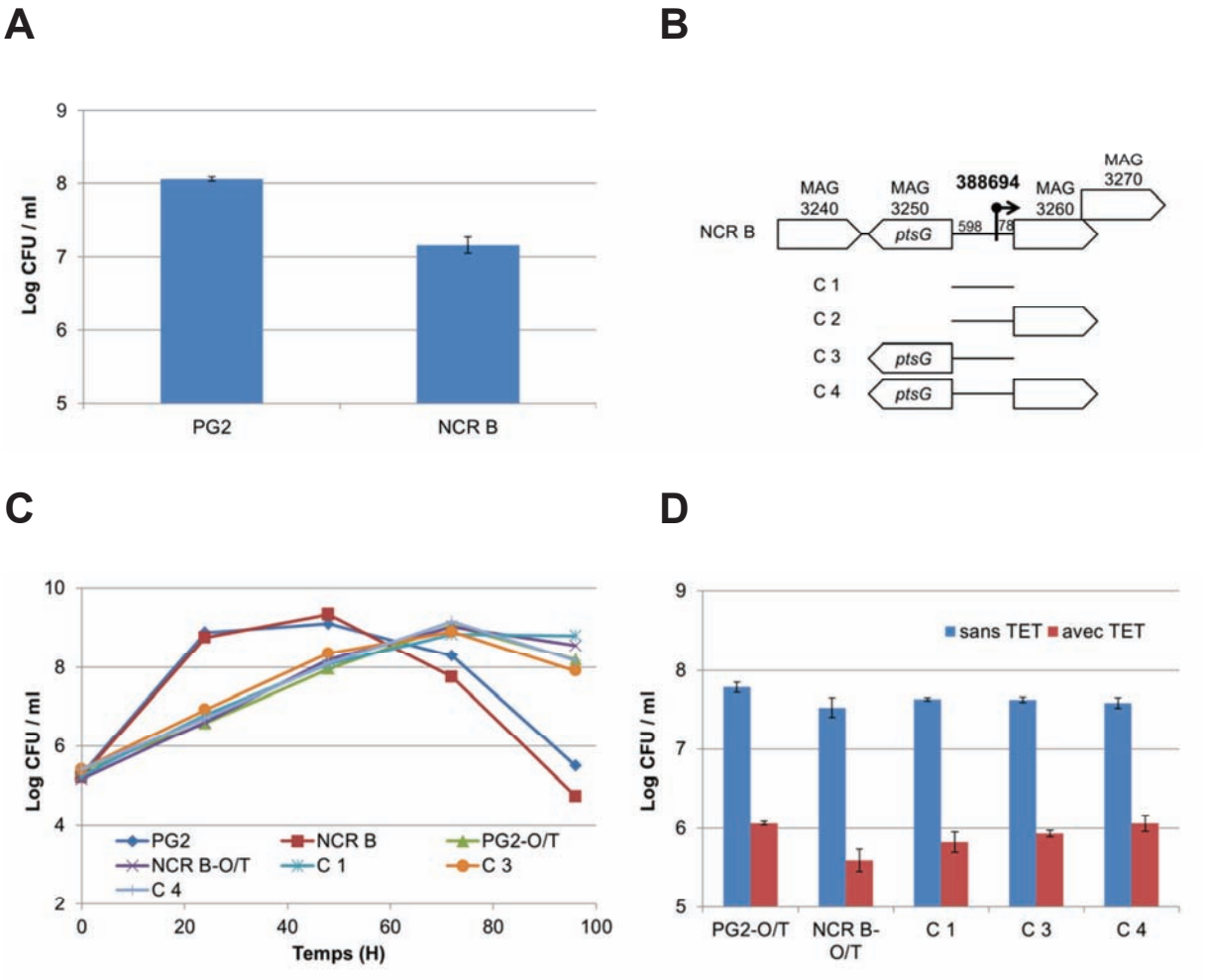


Figure 5. Complémentation du mutant NCR B. (A) Comparaison des titres de la souche PG2 et du mutant NCR B après 72 h de co-culture en présence de cellules TIGMEC ; (B) représentation schématique des quatre constructions utilisées pour la complémentation du mutant NCR B ; (C) courbes de croissance de la souche PG2 (PG2) et du mutant NCR B (NCR B) en milieu axénique, ainsi que de la souche PG2 transformée par le vecteur vide (PG2-O/T), du mutant NCR B transformé par le vecteur vide (NCR B-O/T) ou par les constructions C1 (C1), C3 (C3) ou C4 (C4) ; (D) comparaison des titres de la souche PG2 transformée par le vecteur vide (PG2-O/T), du mutant NCR B transformé par le vecteur vide (NCR B-O/T) ou par les constructions C1 (C1), C3 (C3) ou C4 (C4) après 72 h de co-culture en présence de cellules TIGMEC. Les titrages de mycoplasmes ont été réalisés en présence (avec TET), ou en absence de tétracycline (sans TET).

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Conclusions et Perspectives

CONCLUSIONS ET PERSPECTIVES

La multiplication des approches globales en microbiologie a abouti à l'accumulation d'une masse de données sans précédent. Malgré l'explosion du nombre d'informations sur les génomes et protéomes bactériens, la fonction de nombreux gènes demeure encore inconnue, et l'impact des « omiques » sur la compréhension des mécanismes de virulence reste finalement très limité.

Face à cette situation, les approches fonctionnelles prennent toute leur importance. Au cours de ce travail de thèse, nous avons développé un système de criblage en culture cellulaire qui a permis de mener une étude à haut-débit des interactions entre *M. agalactiae* et les cellules de l'hôte. Ce système fournit un nouvel outil pour l'étude des facteurs de virulence chez les mycoplasmes.

La culture cellulaire ne se substitue pas à l'hôte naturel, mais elle fournit un environnement adapté au criblage massif de banques de mutants et permet d'appréhender, par une approche de génomique fonctionnelle à haut-débit, l'étude des fonctions biologiques qui contribuent à la survie de *M. agalactiae* chez son hôte. Le développement d'un modèle d'infection expérimental chez l'animal reste, à ce stade, incontournable pour valider les données issues du criblage en culture cellulaire. L'évolution rapide des techniques d'analyse à haut-débit et leur adaptation aux particularités des mycoplasmes devraient permettre de s'affranchir du criblage cellulaire et d'envisager, à terme, des études à haut-débit directement chez l'hôte naturel.

La réponse immune de l'hôte est un exemple flagrant des contraintes et des limites liées à la culture cellulaire. L'utilisation de cellules dérivées du système immunitaire et le développement de méthodes de criblage adaptées pourraient permettre de tenir compte de certains éléments de la réponse immune dans l'étude des interactions hôte-mycoplasme. De même, la culture de cellules primaires ou de tissus différenciés, pourrait permettre de contourner d'autres limitations.

La mutagenèse transpositionelle chez les mycoplasmes est limitée à un nombre restreint d'espèces manipulables génétiquement, dont fait partie *M. agalactiae*. Toutefois, les résultats générés chez ce pathogène peuvent être étendus à d'autres espèces proches phylogénétiquement et difficilement manipulables, comme *M. bovis*. L'analyse fonctionnelle du génome d'espèces plus éloignées, grâce notamment à l'amélioration des techniques de

transformation, apportera une vision plus globale des interactions hôte-pathogène au sein des *Mollicutes*.

Le rôle essentiel du locus NIF pour la survie de *M. agalactiae* chez l'hôte naturel souligne la pertinence du criblage cellulaire pour l'étude des facteurs de virulence chez les mycoplasmes. L'autre résultat marquant de cette étude est la découverte, dans le génome de *M. agalactiae,* de plusieurs régions non codantes capables d'influencer la croissance de ce mycoplasme en culture cellulaire. Le rôle de ces régions non codantes dans la biologie des mycoplasmes demeure largement inexploré, mais leur étude pourrait dévoiler de nouvelles pistes pour la compréhension des mécanismes d'expression du pouvoir pathogène chez ces organismes.

Une catégorie de protéines a été largement sélectionnée au cours cette étude. Elle regroupe les protéines de surface qui, en absence de paroi, sont supposées jouer un rôle central dans les interactions entre le mycoplasme et son environnement. Afin d'étudier plus spécifiquement cette catégorie de protéines, les mutants correspondants ont été recherchés au sein de la banque. L'étude de sous-groupe de mutants correspondant à une catégorie fonctionnelle définie permettra de réduire considérablement le volume de mutants et d'envisager un crible plus spécifique en culture cellulaire ou directement chez l'animal.

Les gènes échangés entre *M. agalactiae* et les mycoplasmes du groupe mycoides représentent un autre groupe d'intérêt pour l'étude des interactions hôte-mycoplasme. Ces gènes pourraient avoir un rôle clé dans l'adaptation du mycoplasme à son hôte.

En conclusion, la culture cellulaire combinée à la reproduction expérimentale de la maladie chez l'animal, fournit une stratégie de criblage efficace pour l'identification des facteurs de virulence chez les mycoplasmes et la compréhension de leurs mécanismes de pathogénicité. Ces résultats fournissent également une base expérimentale pour le développement de stratégies vaccinales pertinentes qui tiennent compte des particularités de ces pathogènes.

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Critical Role of Dispensable Genes in *Mycoplasma agalactiae* Interaction with Mammalian Cells▽

Eric Baranowski, Sébastien Guiral,† Eveline Sagné, Agnès Skapski, and Christine Citti*

INRA, UMR 1225, F-31076 Toulouse, France, and Université de Toulouse, Ecole Nationale Vétérinaire de Toulouse, UMR 1225, F-31076 Toulouse, France

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Mycoplasmas are minimal bacteria whose genomes barely exceed the smallest amount of information required to sustain autonomous life. Despite this apparent simplicity, several mycoplasmas are successful pathogens of humans and animals, in which they establish intimate interactions with epithelial cells at mucosal surfaces. To identify biological functions mediating mycoplasma interactions with mammalian cells, we produced a library of transposon knockout mutants in the ruminant pathogen *Mycoplasma agalactiae* and used this library to identify mutants displaying a growth-deficient pheonotype in cell culture. *M. agalactiae* mutants displaying a 3-fold reduction in CFU titers to nearly complete extinction in coculture with HeLa cells were identified. Mapping of transposon insertion sites revealed 18 genomic regions putatively involved in the interaction of *M. agalactiae* with HeLa cells. Several of these regions encode proteins with features of membrane lipoproteins and/or were involved in horizontal gene transfer with phylogenetically distant pathogenic mycoplasmas of ruminants. Two mutants with the most extreme phenotype carry a transposon in a genomic region designated the NIF locus which encodes homologues of SufS and SufU, two proteins presumably involved in [Fe-S] cluster biosynthesis in Gram-positive bacteria. Complementation studies confirmed the conditional essentiality of the NIF locus, which was found to be critical for proliferation in the presence of HeLa cells and several other mammalian cell lines but dispensable for axenic growth. While our results raised questions regarding essential functions in mycoplasmas, they also provide a means for studying the role of mycoplasmas as minimal pathogens.

Often portrayed as minimal bacteria, mycoplasmas have evolved from low-G+C content Gram-positive ancestors by massive losses of genetic material and extensive genome downsizing (37, 44). As a consequence of this reductive evolution, mycoplasmas are lacking a significant number of biological functions found in more complex bacteria and have increased their dependence on the host for many nutrients. The absence of a cell wall, small size, fastidious growth in cell-free environments, and complex requirements for nutrients are among the most emblematic features of these particular organisms (37). Their minute genomes, which for some species are close to the minimal requirements for sustaining autonomous life, are used as experimental platforms to explore the concept of a minimal cell and as a model system for the design of synthetic bacterial genomes (17, 18, 25, 35). While significant progress has been made in understanding the biology of these minimal organisms under laboratory conditions, little is known regarding mycoplasma factors involved in virulence and host interaction. Recent genomic studies indicated that several mycoplasma species have retained sexual competence, a trait that may provide some pathogenic species with a high potential for adaptation (44, 45).

Mycoplasmas are widely distributed in nature, and several species are successful pathogens, capable of establishing persistent infections and causing debilitating diseases in humans and a wide range of animal species (37). Mycoplasmas are also recurrently found associated with cultures of mammalian cells, where they can survive for long periods, often without apparent signs of contamination but with potential consequences for the reliability of experimental results and the safety of biological products. Classified by the World Organization for Animal Health (OIE) as notifiable diseases, a number of mycoplasma infections in domestic animals can have a significant impact on livestock production (16). Among those, the ruminant pathogen *Mycoplasma agalactiae* is the main etiological agent of contagious agalactia in sheep and goats, a syndrome that is characterized by mastitis, arthritis, keratoconjunctivitis, and pneumonia (3). Although phylogenetically distant from *M. agalactiae*, several members of the so-called mycoides cluster, such as *Mycoplasma mycoides* subsp. *mycoides* large colony (MmmLC) and *Mycoplasma capricolum* subsp. *capricolum* (Mcc), are also able to induce similar symptoms in the same ruminant species. Remarkably, whole-genome sequence analysis has revealed that extensive horizontal gene transfer (HGT) events, affecting up to 18% of the *M. agalactiae* genome, occurred between *M. agalactiae* and members of the mycoides cluster, illustrating the unexpected plasticity and adaptability of the mycoplasma genome (44, 45).

Recent advances in whole-genome sequencing have greatly facilitated the study of mycoplasmas (2, 33, 34, 44, 45). Unfortunately, these data alone have been of little help in identifying the mechanism underlying diseases caused by mycoplasmas. The main reason is that the predicted mycoplasma gene products, other than those involved in housekeeping functions, display little

* Corresponding author. Mailing address: UMR 1225 INRA, Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, BP 87614, 31076 Toulouse cedex 3, France. Phone: 33 5 6119 38 56. Fax: 33 5 6119 3273. E-mail: c.citti@envt.fr.

† Present address: Institut de Biologie et Chimie des Protéines, CNRS, Université de Lyon, 7, passage du Vercors, 69367 Lyon Cedex 07, France.

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or no homology to those known for classical bacteria (45). Among the few exceptions is the ADP-ribosylating cytotoxin found in the human respiratory pathogen *Mycoplasma pneumoniae* that displays some similarity with the pertussis toxin (27).

Factors that may contribute to the pathogenic process in mycoplasmal infections include the capacity to adhere and invade host cells, the production of immunomodulatory molecules, and a highly variable antigenic structure, as well as the formation of biofilm and the release of metabolic hydrogen peroxide (5, 10, 22, 29, 41). Gene products presumably involved in *M. agalactiae*-host interaction include the P40 adhesion protein (13), a family of phase-variable surface proteins, designated Vpma, which are encoded by a locus subjected to high-frequency DNA rearrangements (19, 20), and the immunomodulatory P48 protein (40).

Transposon mutagenesis has been used extensively as a tool for the identification of virulence genes in pathogenic bacteria. Similar approaches have been developed with a few mycoplasma species, mainly with the aim of defining the minimal set of essential genes required to sustain autonomous life under axenic conditions (12, 15, 18, 25). However, the genetic information necessary to develop interactions of mycoplasma with its animal host is likely to differ from the minimal set of essential genes required for laboratory growth. New opportunities to investigate factors involved in mycoplasma-host interaction have emerged through the development of genomic tools that facilitate the manipulation of animal mycoplasmas including *M. agalactiae* (8, 9). However, *in vivo* screening of mutant libraries of ruminant mycoplasmas involves difficulties inherent in experiments with infections in large animals.

The present study combines large-scale transposon mutagenesis of the pathogen *M. agalactiae* and an appropriate model of bacteria-HeLa cell interactions in coculture. Genomic regions of *M. agalactiae* specifically required for survival under cell culture conditions, while dispensable for axenic growth, were thus identified, indicating that mycoplasma cocultivation with mammalian cells represents an original and efficient system for high-throughput screening of large mutant libraries.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. agalactiae* reference strain PG2 (45) was cultured at 37°C in Aluotto or SP4 medium (46) supplemented with 500 μg/ml cefalexin (Virbac). Gentamicin (50 μg/ml) was added to the medium for the propagation of *M. agalactiae* mutants generated by transposon mutagenesis. Mycoplasma cultures were stored at −80°C as 10-μl aliquots. CFU titers were determined by serial dilutions in Dulbecco's phosphate-buffered saline (Invitrogen) supplemented with 1% heat-inactivated horse serum (Invitrogen). Dilutions were spotted (10 μl) onto solid Aluotto or SP4 medium, and mycoplasma colonies were counted after 2 to 5 days incubation at 37°C. *E. coli* DH10B [F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lac*ZΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL endA1 nupG*] (Invitrogen) was used for DNA cloning and plasmid propagation. *E. coli* bacteria were grown in Luria broth supplemented with ampicillin (50 μg/ml) and/or tetracycline (5 μg/ml) when required. Sheep and horse erythrocytes were derived from defibrinated blood (bioMérieux).

Plasmids and DNA constructions. Plasmid pMT85, which contains a modified version of transposon Tn*4001* (mini-Tn), was kindly provided by Richard Herrmann (48). The gentamicin resistance marker encoded by the *aacA-aphD* gene is located between the two inverted repeats (IRs) that define the extremities of the transposed fragment, while the transposase gene (*tnpA*) is located outside the transposable elements to prevent reexcision events. Plasmid p20-1miniO/T was used as a shuttle vector for complementation studies of *M. agalactiae*. Plasmid p20-1miniO/T was derived from pMM20-1 (8) by partial deletion of both the *tetM* region and the 6.9-kb DNA fragment carrying the *M. agalactiae* origin of replication. For complementation of *M. agalactiae* mutants T07.082 (clone 82 isolated from transformation T07) and T07.134, the genomic region designated the NIF locus (MAG0720 and MAG0730) was cloned downstream of the P40 gene promoter region. These two regions were assembled by PCR amplification using an overlapping primer. The promoter region was amplified first by using oligonucleotide primers p40RF-CC (5′-ACGGGGCTAAAGAAGCTGAT-3′) and P40-nifS-R (5′-GATCTAATCGATTTAGGCATAATTATTTATATCCTT TTC-3′) to generate a 200-bp DNA fragment overlapping coding sequence (CDS) MAG0720 at the ATG codon. The NIF locus was then amplified by using the overlapping DNA fragment and the primer 88595_R (5′-CTGTGCGCGCT TACAAAGTA-3′). The resulting PCR product was cloned into pGEM-T Easy (Promega) before subcloning at the NotI site of the p20-1miniO/T plasmid to generate pO/T-NIF. Cloned sequences were verified by DNA sequencing. PCRs were performed using an Expand High Fidelity PCR System (Roche).

Cell lines. Human epithelial HeLa cells (ATCC CCL2; cervical carcinoma) were kindly provided by P. Mason (University of Texas Medical Branch, Galveston, TX). The bovine turbinate cells (BT; ATCC CRL-1390) were purchased at the ATCC. The bovine cell line KOP (esophageal tissue of a calf) was obtained from the Friedrich Loeffler Institute (Greifswald-Insel Riems, Germany). The caprine cell lines including simian virus 40 (SV40) large T-antigen immortalized goat embryo fibroblasts (TIGEF) (11) and similarly immortalized milk epithelial cells (TIGMEC) (31) were kindly provided by C. Leroux (INRA, Lyon, France). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)-based medium. This medium is composed of DMEM (high glucose, sodium pyruvate, and GlutaMAX-I; Invitrogen) supplemented with nonessential amino acids (NEAA; Invitrogen) and 10% heat-inactivated fetal calf serum (FCS; Eurobio). Cells were incubated at 37°C in an atmosphere with 5% CO_2 and subcultured every 2 to 3 days by seeding one-third to one-sixth the number of cells reached at confluence.

Cocultivation of *M. agalactiae* with mammalian cells. Cocultivations were carried out in DMEM-based medium, supplemented with gentamicin (50 μg/ml), for *M. agalactiae* mutants. Since Aluotto broth (up to 0.1%) has no apparent toxic effect on mammalian cells (data not shown), mycoplasma inocula were prepared by direct dilution of frozen mycoplasma cultures in DMEM-based medium. Mammalian cells were prepared by trypsin-EDTA treatment of nearly confluent monolayers. After low-speed centrifugation, cells were resuspended in DMEM-based medium, seeded in 24-well plates (Falcon) at a density of 2×10^4 cells/cm^2, and inoculated with *M. agalactiae* at different multiplicities of infection (MOI). Mycoplasma and mammalian cells were then allowed to grow at 37°C under 5% CO_2. At different times postinoculation, mycoplasma titers were determined by CFU titrations following one freeze-thaw (−80°C/+37°C) cycle to disrupt mammalian cells.

Transformation of *M. agalactiae* with plasmid DNA. Transformation of mycoplasma cells (10^8 to 10^9 CFU) was performed by electroporation using 1 to 3 μg of plasmid DNA, as described previously (9). Following electroporation, mycoplasma cells were incubated in nonselective SP4 medium for 3 h at 37°C. Cells were then allowed to grow in the presence of the appropriate antibiotic for an additional period of 3 to 12 h before they were plated on selective SP4 agar. Gentamicin and tetracycline were used at a concentration of 50 μg/ml and 2 μg/ml, respectively. Isolated colonies were picked after 4 to 7 days, and transformants were subcultured in 1 ml of selective SP4 medium.

Transposon mutagenesis in *M. agalactiae*. Transposon mutagenesis in *M. agalactiae* was carried out using plasmid pMT85. Colonies were collected from independent transformations and subcultured in 1 ml of selective SP4 medium. Cultures of individual mutants were distributed in 96-well plates, and the pMT85-based library was stored at −80°C. Transposon insertion sites in the *M. agalactiae* chromosome were mapped by sequencing the junction between *M. agalactiae* genomic DNA and the 3′ end of the transposon, using the orientation of the gentamicin resistance gene as a reference. Genomic DNA (5 μg) was sequenced using BigDye terminator chemistry and oligonucleotide SG8 (5′-GA GTCAGTGAGCGAGGAAGC-3′) as a primer. Direct sequencing of genomic DNA was performed at the sequencing facility of the Bio-Medical Research Federative Institute of Toulouse (Toulouse, France).

Identification of growth-deficient mutants under coculture conditions. A 96-pin replicator (Boekel Scientific) was used for high-throughput screening of the library and the identification of mutants displaying reduced growth capacity under cell culture conditions. Cocultivation of individual mutants with HeLa cells was performed in 96-well plates. Cells were seeded in DMEM-based medium at a density of 2×10^4 cells/cm^2 and inoculated with individual mutants using the 96-pin replicator. The volume of the sample transferred by one pin of the replicator was estimated at about 1 μl. After 3 days of cocultivation, plates were submitted to one freeze-thaw (−80°C/+37°C) cycle and spotted onto solid medium using the 96-pin replicator. The development of mycoplasma colonies was observed after 5 to 10 days of incubation at 37°C. Growth-deficient mutants

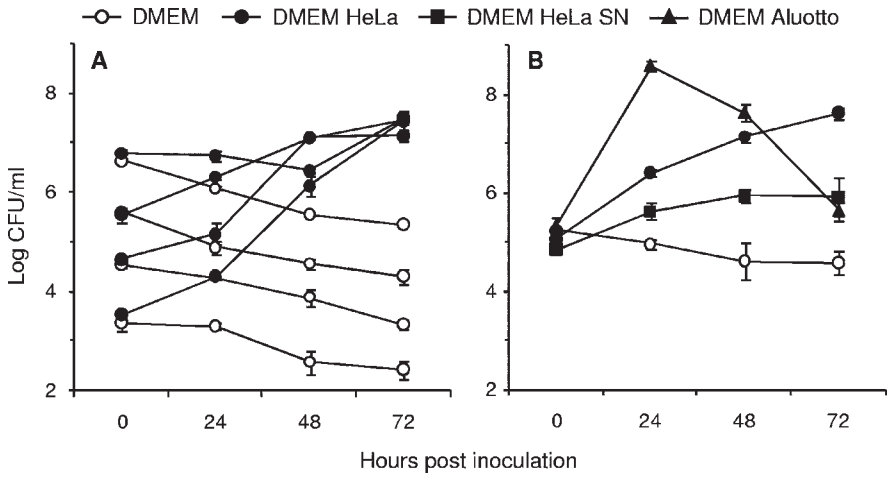


FIG. 1. Growth and survival of *M. agalactiae* under cell culture conditions. Serial dilutions from mycoplasma stocks (A) or defined dilutions (B) were inoculated to HeLa cells seeded at a density of 2×10^4 cells per cm^2 in DMEM-based medium (DMEM HeLa), DMEM-based medium alone (DMEM), DMEM-based medium preincubated with HeLa cells (DMEM HeLa SN), or DMEM-based medium supplemented with 10% Aluotto broth (DMEM Aluotto). Cultures were incubated at 37°C under 5% CO_2, and mycoplasma titers (log CFU/ml) were determined by CFU titrations following one freeze-thaw cycle of to disrupt HeLa cells. The data are the means of at least three independent assays. Standard deviations are indicated by error bars.

failed to produce detectable numbers of CFU upon cocultivation with HeLa cells. The presence of viable CFU in culture stocks of *M. agalactiae* mutants was controlled by direct spotting onto solid medium.

PCR-based screening of the mutant library. The detection of mutants with transposon insertion events at specific genomic regions in the whole library was performed by PCR amplification using genomic DNA prepared from individual mutants or mutant pools containing up to 96 individual mutants. Mutant pools were constituted by the addition of a 15- to 30-μl aliquot of each mutant culture. Mutants with a transposon inserted at genomic position 180349 (MAG1540) were identified using the *M. agalactiae*-specific primer 181025_TIG_R (5′-TCT CCACAGGAACAGTTGCTTA-3′), which spans genomic positions 181025 to 181004, and the transposon-specific oligonucleotide SG8 (5′-GAGTCAGTGA GCGAGGAAGC-3′) priming at the 3′ end of the integrated transposon sequence. PCR amplifications (25 μl) were performed according to the recommendations of the *Taq* DNA polymerase supplier (New England Bioloabs).

Southern blot hybridization. Genomic DNA (1 μg) was digested by HindIII, and hybridization was performed in the presence of digoxigenin (DIG)-labeled DNA probes as described previously (28).

RESULTS

Proliferation of *M. agalactiae* in cell culture depends on HeLa cells for nutrients. To assess the capacity of *M. agalactiae* to proliferate under cell culture conditions, HeLa cells were infected with serial dilutions of mycoplasma cultures, and the growth of mycoplasmas over a period of 72 h was determined by enumerating CFU. Unless incubated with HeLa cells, *M. agalactiae* was unable to grow in DMEM-based medium alone, despite supplementation with fetal bovine serum (Fig. 1A). HeLa cells dramatically enhanced *M. agalactiae* proliferation under cell culture conditions, yet CFU titers were about 100-fold lower than those produced in axenic media, which are classically used to propagate *M. agalactiae* under laboratory conditions (data not shown). Conversely, *M. agalactiae* had no visible effect on cell monolayer development, at least during the first 72 h of infection (data not shown). At the end of the incubation period, *M. agalactiae* reached an average titer of ca. 10^7 CFU/ml, with a mycoplasma/cell ratio estimated at about 50 to 100 bacteria per cell, regardless of the starting inoculum. This offered the possibility to compare simultaneously a large collection of individual mutants of unknown CFU titers, as performed below.

The growth-promoting effect of HeLa cells on *M. agalactiae* was further examined by using a cell culture medium preincubated with HeLa cells or supplemented with Aluotto broth. As shown in Fig. 1B, *M. agalactiae* growth was observed under both conditions, indicating that a deficiency in essential nutrients, rather than the presence of growth inhibitors, was probably responsible for the absence of proliferation in DMEM-based medium. This suggests that HeLa cells may provide a number of nutrients or growth factors that are required for *M. agalactiae* proliferation under cell culture conditions.

Although human epithelial surfaces are probably not a natural environment for *M. agalactiae*, these results indicate that HeLa cells may provide a useful model system to study basic interactions of mycoplasmas with mammalian cells.

Isolation of *M. agalactiae* growth-deficient mutants in cell culture. An *M. agalactiae* library of 1,813 gene-disrupted mutants was generated by transposition mutagenesis using plasmid pMT85 (48), which does not replicate in mycoplasma and contains a modified version of transposon Tn*4001* (mini-Tn). Because in this plasmid the transposase gene has been placed outside the transposon, the random insertion of the mini-Tn in the mycoplasma genome is stable in addition to conferring gentamicin resistance. Given the low transformation efficiency of *M. agalactiae*, mutants were collected from multiple individual transformations to produce a representative library (Table 1).

A high-throughput screening strategy was developed to identify mutants displaying a growth-deficient pheontype in cell culture. Using this approach, a first set of 23 mutants was selected (group A) whose multiplication in cell culture failed to produce detectable titers (with a detection limit of about 10^4 to 10^5 CFU/ml); a second set of 176 mutants (group B) displaying apparently reduced, but still detectable, CFU production was also selected. The distribution of these mutants obtained from independent transformation events ranged from 0 to 5% for

TABLE 1. Transposon mutagenesis in *M. agalactiae*

Transformation no.[a]	No. of clones isolated	No. (%) of mutants in:	
		Group A[b]	Group B[c]
T01	52	0 (0.0)	5 (9.6)
T02	65	0 (0.0)	8 (12.3)
T05	149	8 (5.4)	30 (20.1)
T06	169	3 (1.8)	35 (20.7)
T07	175	2 (1.1)	1 (0.6)
T08	270	2 (0.7)	23 (8.5)
T09	247	1 (0.4)	47 (19.0)
T10	686	7 (1.0)	27 (3.9)
Total	1,813	23 (1.3)	176 (9.7)

[a] Independent transformation assays with plasmid pMT85.
[b] Growth-deficient mutants in cell culture.
[c] Mutants found to be moderately inhibited upon high-throughput screening of the mutant library in cell culture.

group A and from 1 to 21% for group B (Table 1) of the total number of transformants.

In this study, only mutants of group A were further examined because of their marked phenotypes. After subcloning, the growth phenotype of each mutant was determined under both axenic and cell culture conditions (Fig. 2). Compared to the parental strain PG2 or to the control mutant, namely, T08.101, these mutants displayed a 3-fold reduction in CFU titers to nearly complete extinction in the presence of HeLa cells while producing wild-type CFU titers in Aluotto broth. One exception was mutant T05.137, whose multiplication was affected under both cell culture and axenic conditions.

Mapping of transposon insertion sites in *M. agalactiae* growth-deficient mutants. Direct sequencing of genomic DNA from group A mutants revealed single transposon insertion events in each mutant and identified 19 insertion sites mapping within 15 different coding sequences (CDSs), and three noncoding regions (NCR) (Fig. 2). CDSs found to be disrupted in group A mutants corresponded to proteins belonging to a broad spectrum of COG (cluster of orthologous groups) functional categories including chaperones (trigger factor; O-COG0544), nucleotide metabolism (adenine phosphoribosyltransferase; F-COG0503), amino acid metabolism (nitrogen fixation protein NifS; E-COG0520), and DNA repair (UvrABC system protein A; L-COG0178), as well as proteins with poorly characterized functions (transport protein SGAT [S-COG3037], esterase/lipase [R-COG0596], and GTPase EngC [R-COG1162]) and hypothetical proteins (HP) of unknown functions, some of which have features of membrane-bound lipoproteins.

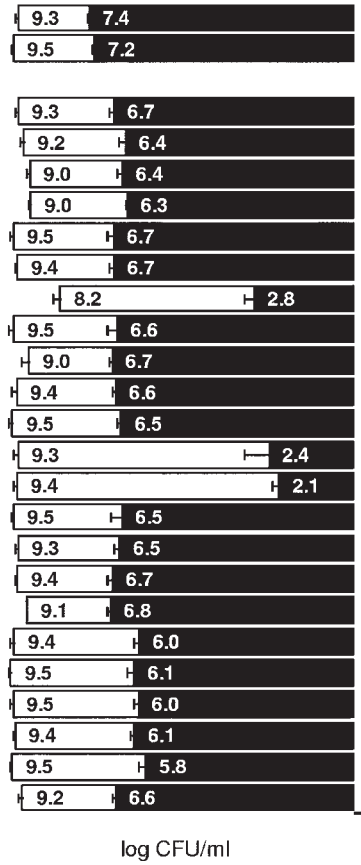
Localization of transposon insertion sites in the *M. agalactiae* chromosome of group A mutants failed to reveal any hot spot for the transposition of mini-Tn. However, three mutants (13% of the total number of selected mutants) were found to have a transposon inserted within a 20-kb locus containing a vestige of an integrative conjugative element, ICEA, encompassing CDS MAG4060 to MAG3860 in *M. agalactiae* strain PG2 (45). Insertions were found in pseudogene MAG3880 (mutant T06.016) and two NCR located upstream of MAG4010 (mutant T08.070) and downstream of MAG3960 (mutant T05.099). Reexamination of the *M. agalactiae* genome using AMIGene software (6) predicted the presence of a short CDS (spanning genomic position 469474 to 469319) which was disrupted in mutant T05.099 and was not previously annotated.

A detailed analysis of transposition events in group A mutants revealed an important bias in the orientation of the integrated mini-Tn sequence. Using the mini-Tn gentamicin resistance gene as a reference (48), almost all CDSs found disrupted were harboring a transposon inserted in an antisense direction relative to the transcription of the mutated CDS. One exception was mutant T07.134, which has a positively oriented mini-Tn in CDS MAG0720 (see below). The *lac* promoter and the promoter of the gentamicin resistance-encoding gene are the only regulating sequences provided by the mini-Tn. Both are transcribed in the same direction (48). Experiments are in progress to determine whether transcription from these two promoter regions can extend beyond mini-Tn-inserted sequences and influence the orientation of the integrated mini-Tn sequence.

Several growth-deficient mutants identified by our screening strategy shared identical insertion sites (Fig. 2). As expected, mutants with identical insertions produced similar CFU titers under cocultivation conditions, suggesting that these mutants were probably siblings derived from the same parental clone. To rule out a possible bias in our screening strategy due to the outgrowth of mutants with higher degrees of fitness, we used a PCR-based screening method to determine the frequency of mutants sharing identical insertion sites. Analysis of the 149 mutants derived from transformation T05 identified two mutants with a transposon inserted at genomic position 180349 (MAG1540). These two mutants were those selected upon cocultivation with HeLa cells, which indicates that the screening procedure was efficient.

The NIF locus is essential for *M. agalactiae* growth under cell culture conditions but is dispensable for axenic growth. Two mutants (T07.082 and T07.134) with the most extreme phenotypes under coculture conditions have a transposon inserted in a locus composed of two genes with homology to nitrogen fixation proteins NifS and NifU (MAG0720 and MAG0730). The insertion site identified in mutant T07.082 was found to disrupt the *nifS* gene at a region corresponding to the N-terminal part of the protein (Fig. 2). Mutant T07.134 had a transposon inserted within the same gene, right into the stop codon (TAG). Interestingly, in this mutant the mini-Tn insertion is such that it restored the sequence of the termination codon, suggesting that the phenotype of mutant T07.134 cannot be explained simply by disruption of the gene encoding the NifS protein.

In contrast to the situation described in *Mycoplasma genitalium* (18, 25), the ability to obtain such mutants suggests that the NIF locus (MAG0720 and MAG0730) is dispensable for *M. agalactiae* growth under axenic conditions. The dispensability of the NIF locus for axenic growth of mutants T07.82 and T07.134 was further confirmed in Aluotto and SP4 media, both of which are classically used to propagate *M. agalactiae* under laboratory conditions (data not shown). BLAST analysis of the *M. agalactiae* genome sequence failed to reveal an additional copy of CDS MAG0720 or the presence of a paralog, ruling out the possibility of an insertion event having inactivated one copy of a duplicated *nifS* gene in mutants T07.82 and T07.134. However, essential genes can also be found disrupted if gene products are supplied by other mutants in mixed populations.



Mutant	Genomic position	CDS	CDS position	CDS identity and comments
Parental strain and control mutant				
PG2				Parental strain
T08.101	552034 (-)	MAG4720	0.66 (-)	Control mutant
Group A mutants				
T05.005	699733 (+)	MAG6010	0.24 (-)	HP
T05.081	603060 (-)	MAG5150	0.91 (-)	HP
T05.084	180349 (+)	MAG1540	0.27 (-)	Trigger factor (tig)
T05.094	= T05.084			
T05.098	355485 (+)	MAG2960 *	0.17 (-)	CHP, predicted lipoprotein
T05.099	469389 (+)	NCR		Downstream (39n) of MAG3960 *, CHP; part of ICEA
T05.137	811528 (+)	MAG6960	0.58 (-)	Adenine phosphoribosyltransferase (*apt*)
T05.143	413276 (+)	MAG3480	0.56 (-)	HP
T06.006	706544 (+)	MAG6060	0.36 (-)	HP, predicted lipoprotein
T06.016	459971 (+)	MAG3880 *	0.02 (-)	Pseudogene of CHP, C-terminal part ; part of ICEA
T06.072	754389 (+)	MAG6380	0.91 (-)	Transport protein SGAT
T07.082	087172 (-)	MAG0720	0.18 (-)	Nitrogen fixation protein NifS (*nifS*)
T07.134	088125 (+)	MAG0720	1.00 (+)	Nitrogen fixation protein NifS (*nifS*)
T08.055	004610 (-)	MAG0040 *	0.79 (-)	Esterase/lipase
T08.070	473081 (+)	NCR		Upstream (61n) of MAG4010, HP; part of ICEA
T09.075	450468 (-)	MAG3790	0.95 (-)	uvrABC system protein A (*uvrA*)
T10.168	150813 (+)	MAG1330 *	0.31 (-)	CHP, DUF285 family
T10.277	388694 (+)	NCR		Upstream (79n) of MAG3260 *, CHP, DUF285 family
T10.326	397462 (+)	MAG3350	0.82 (-)	HP
T10.452	245446 (-)	MAG2130	0.75 (-)	GTPase EngC (*engC*)
T10.528	= T10.277			
T10.540	= T10.452			
T10.645	= T10.168			

FIG. 2. Characterization of *M. agalactiae* mutants displaying altered growth in cell culture. Mutants were designated according to transformation and clone numbers (e.g., T05.081 designates clone 81 isolated from transformation T05). PG2 and T08.101 refer to the parental strain and the control mutant, respectively. Group A mutants were selected from the mutant library by high-throughput screening on HeLa cells, as described in the Results section. Mycoplasma titers (log CFU/ml) at 48 h in Aluotto broth (open bars) and at 72 h in cell culture (solid bars) are compared. The data are the means of at least three independent assays. Standard deviations are indicated by error bars. Transposon insertion sites were determined by direct sequencing of genomic DNA, and their positions were defined based on the published sequence (NC_009497). The orientation of the inserted sequences is indicated in parenthesis. Mutants sharing an identical insertion are indicated. CDSs found disrupted in *M. agalactiae* mutants are indicated by the mnemonic codification (45). Noncoding regions are indicated. CDSs involved in horizontal gene transfer between *M. agalactiae* and mycoplasmas of the mycoides cluster (45) are designated by asterisks (*). For each CDS, the relative position and orientation of the inserted transposon are indicated (CDS position). Hypothetical proteins (HP) have no homologs outside the *M. agalactiae* species. Conserved hypothetical proteins (CHP) share sequence similarity with proteins of unknown function identified in mollicutes or other bacteria. Several insertion sites mapped within a 20-kb locus that contains a vestige of an integrative conjugative element (ICEA).

To rule out this hypothesis, the presence of wild-type sequences in cloned *nifS* mutants propagated in medium without gentamicin was tested by PCR assay using oligonucleotide primers flanking the transposon insertion site in mutants T07.82 and T07.134 (Fig. 3). The absence of wild-type sequences was confirmed in all populations tested. Finally, Southern blotting performed on *nifS* mutant populations at passages 1 to 20 in selective or nonselective medium confirmed the stability of the inserted sequences (Fig. 3).

The growth phenotype of *nifS* mutants T07.82 and T07.134 under cell culture conditions was further characterized. Growth experiments were carried out using HeLa cells and a number of animal cell lines derived from ruminant species (see Materials and Methods). Incubation with HeLa cells, while producing a growth-promoting effect on the *M. agalactiae* parental strain and control mutant T08.101, had the opposite effect on *nifS* mutants T07.82 and T07.134 (Fig. 4). This dual effect, growth-promoting and death-inducing, was not restricted to HeLa cells and was also observed with all mammalian cell lines tested in this study, although to different degrees (Fig. 4). These results suggest that components, most likely nutrients released by mammalian cells, were required for *M. agalactiae* proliferation in DMEM-based medium but were toxic for the *nifS* mutants. This was further confirmed by reproducing this dual effect in the absence of mammalian cells, using a DMEM-based medium preincubated with HeLa cells (data not shown).

Finally, disruption of the NIF locus was also found to affect *M. agalactiae* growth in the presence of erythrocytes. Whereas *M. agalactiae* development on blood agar plates produced he-

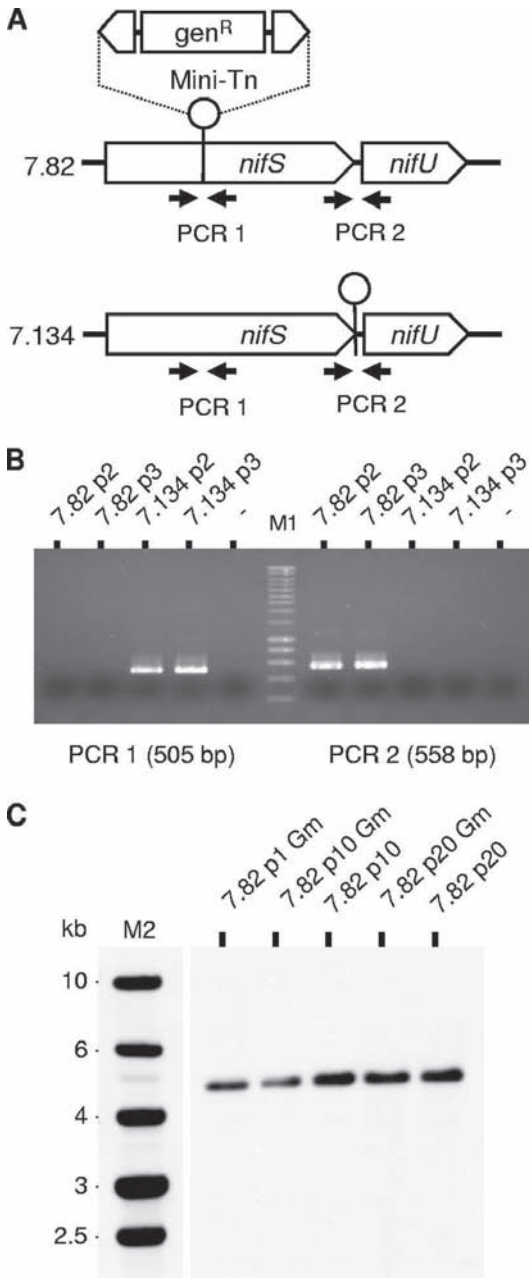


FIG. 3. Clonality and stability of *M. agalactiae* NIF mutant populations. (A) The genomic regions surrounding the transposon insertion site in mutants T07.82 and T07.134 were analyzed by PCR amplification to detect the presence of contaminating transposon-free sequences. PCR1 and PCR2 were performed using the primer pair 86768F (5′-TCAGCC GACATTATTCATGG-3′) and 87272R (5′-CACCGGCTTTTAATTTT TGC-3′) and the pair 88037F (5′-AGGGTTTCGCTAGGGGTTTA-3′) and 88595R (5′-CTGTGCGCGCTTACAAAGTA-3′), respectively. (B) The PCR1 product (505 bp) amplified from mutant T07.134 populations at passages 2 (7.134p2) and 3 (7.134p3) was not detected upon amplification of the corresponding populations of mutant T07.82 (7.82p2 and 7.82p3). The opposite result was observed for the PCR2 product (558 bp). These negative results suggest the absence of detectable contaminating sequences in all the populations tested. M1, molecular weight markers (SmartLadder; Eurogentec). (C) Southern blot analysis of genomic DNA derived from NIF mutant T07.82 (7.82) at passages 1, 10, and 20 in selective (Gm) or nonselective medium. Genomic DNA was digested by HindIII and Southern blot hybridized with DIG-labeled amplicons derived from plasmid pMT85. M2, molecular weight markers (1 Kb DNA ladder; Promega).

molytic zones surrounding colonies, the development of the *nifS* mutants T07.82 and T07.134 was inhibited in a dose-dependent manner by erythrocytes or erythrocyte lysates (Fig. 5) but not by erythrocyte ghosts (data not shown). This inhibition was not species specific since similar results were obtained using horse or sheep erythrocytes. Attempts to further characterize the *nifS* mutant growth-inhibiting factors present in erythrocyte lysates failed. These results further illustrate the vulnerability of the *nifS* mutants when they are exposed to mammalian cells and the critical role played by the NIF locus for *M. agalactiae* survival under these conditions.

Gene complementation studies. Plasmid p20-1miniO/T was used as a shuttle vector for gene complementation studies in *M. agalactiae*. Mutants T07.82 and T07.134 were transformed with the same plasmid in which the NIF locus was introduced (pO/T_NIF). More specifically, the DNA region encompassing the *nifS* and *nifU* genes of PG2 was cloned in p20-1miniO/T downstream of the P40 protein promoter region (see Materials and Methods). The growth of mutants T07.82 and T07.134 on blood agar plates was restored upon transformation with construct pO/T_NIF but not with control plasmid p20-1miniO/T (Fig. 5). Complementation of these two mutants was also confirmed in coculture with mammalian cells (Fig. 5). Growth experiments failed to reveal differences between the parental strain PG2 and complemented mutants. As expected, the phenotype displayed by mutants T07.82 and T07.134 under cocultivation conditions remained unchanged following transformation with the control plasmid p20-1miniO/T. Complementation studies confirmed the conditional essentiality of the NIF locus for *M. agalactiae* under all of these specific culture conditions.

DISCUSSION

The development of axenic culture conditions has considerably facilitated the study of pathogenic mycoplasmas under laboratory conditions. Yet limited information is available regarding the factors that are involved in their virulence and in their interaction with the host, mainly because of the lack of cellular or small-animal models. In an attempt to fill this gap, a model system using the HeLa human epithelial cell line was developed to study the basic interactions between *M. agalactiae* and eukaryotic cells. We further used this model system, combined with the production of a large mutant library, in a high-throughput screening strategy for the identification of *M. agalactiae* growth-deficient mutants and mapped 18 regions on the *M. agalactiae* chromosome that are specifically required for optimal proliferation under cell culture conditions but are dispensable for propagation in axenic medium.

The number of mutants tested was 2.4 times the total number of CDSs found in the genome of the PG2 strain (877 kb; 742 CDSs). Whether the mutant library produced in *M. agalactiae* may be approaching saturation is unknown; however, the number of mutants collected is in agreement with the experimental estimations of the minimal size of a mutant library to reach saturation mutagenesis of all nonlethal insertion sites in other mycoplasma species (15, 18). In the human urogenital pathogen *M. genitalium* (580 kb; 475 CDSs), the number of unique transposon insertion sites drops dramatically after 600 mutants, corresponding to 1.3 times of the total number of CDSs found in this organism; whereas in the murine

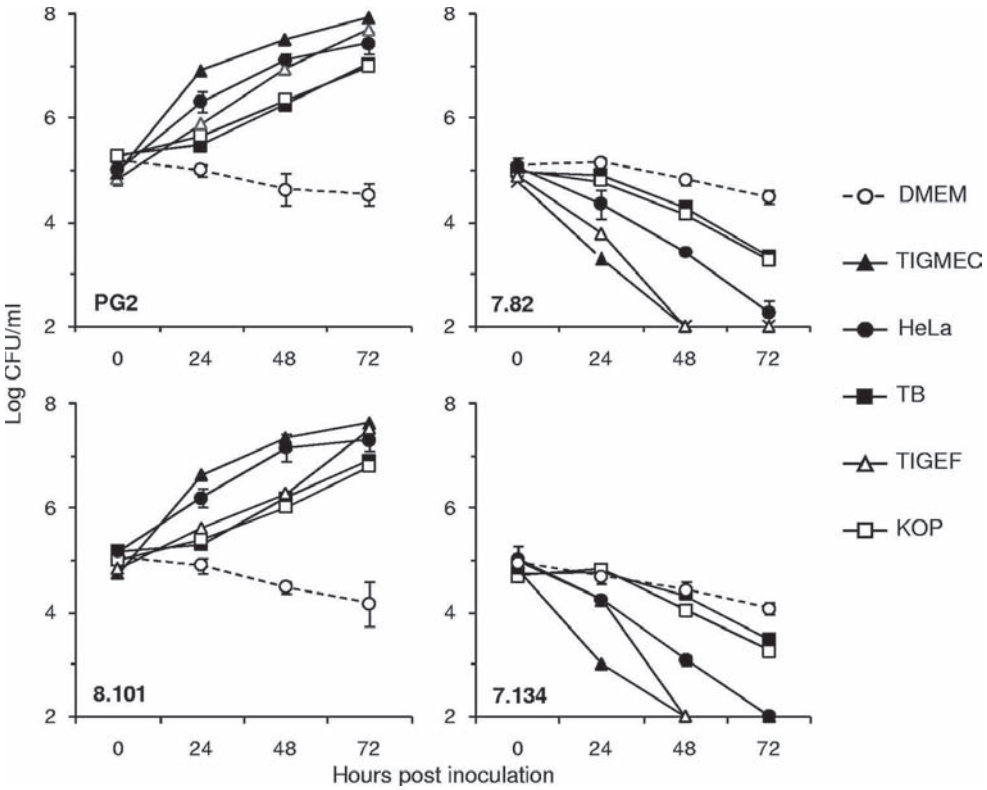


FIG. 4. Comparative growth of *M. agalactiae* NIF mutants under cell culture conditions. *M. agalactiae* parental strain PG2, the control mutant T08.101 (8.101), and NIF mutants T07.082 (7.82) and T07.134 (7.134) were assessed for survival over a 72-h incubation in DMEM-based medium (DMEM) or in coculture with a number of mammalian cell lines including HeLa, goat fibroblast (TIGEF), goat epithelial (TIGMEC), bovine turbinate (TB), and bovine esophageal (KOP) cells. The data are the means of two or three independent assays. Standard deviations are indicated by error bars.

pathogen *M. pulmonis* (964 kb; 782 CDSs), the number of inactivating insertions in genes larger than 1 kb nearly reached a plateau at around 1,800 insertion sites (2.3 times the total number of CDSs). High-throughput screening of the *M. agalactiae* library using the cell system developed here identified a series of 23 mutants displaying a 3-fold reduction in CFU titers to nearly complete extinction in the presence of HeLa cells. The efficiency of this screening was confirmed by (i) the identification of several growth-deficient mutants sharing identical insertion sites, such as mutants T05.084 and T05.094 that were present at low frequency (<1.4%) in the mutant population originating from one transformation event, and (ii) the identification of growth-deficient mutants having a transposon inserted in the same CDS but at different positions.

The availability of the annotated genome sequence of *M. agalactiae* allowed rapid mapping of the transposon insertion sites of selected mutants. Disrupted CDSs for which a predicted function was assigned correspond to 40% of the total number of CDSs and belong to a broad number of functional categories, often with no obvious correlation between the predicted function and its potential role in *M. agalactiae* survival under cell culture conditions. Yet several related functions, such as protein folding, iron-sulfur cluster biosynthesis, and DNA repair, have been associated with virulence or stress tolerance in a number of pathogenic bacteria (1, 4, 24, 39, 42, 43, 47). Another 40% of CDSs encode hypothetical products, many of which were shown to display membrane lipoprotein features and/or to have undergone horizontal gene transfer (HGT) with the mycoides cluster (Fig. 2). Two of these proteins belong to a gene family, the *drp*, which encodes related proteins containing one or several DUF258 domains. This domain is of unknown function and is found in some bacteria but not in mollicutes, with the exception of the mycoides cluster (45). In a recent study comparing *M. agalactiae* strains using whole-genomic and proteomic approaches, the differential expression of some *drp* genes was found in association with the membrane fraction. These data suggested that this family may participate in generating surface diversity, with some *drp* genes presenting features of lipoproteins and being expressed and others serving as sequence reservoirs (34). Interestingly, the *drp* genes are part of the gene pool which has undergone HGT with members of the phylogenetically distant mycoides cluster. This cluster contains only ruminant-pathogenic mycoplasma species, and in the absence of a cell wall, surface-exposed lipoproteins may play an important role in mediating interactions with the host. Further studies are needed to confirm the role of these CDSs in the *M. agalactiae* interaction with mammalian cells, but they provide an interesting subset of mutants that can reasonably be analyzed *in vivo*. Remarkably, a number of integration events occurred in pseudogenes or in NCR that map within a particular 20-kb locus containing a vestige of an integrative conjugative element, ICEA. The implication of an ICEA-related open reading frame (ORF) in the *M. agalactiae* interaction with HeLa cells remains to be confirmed. However,

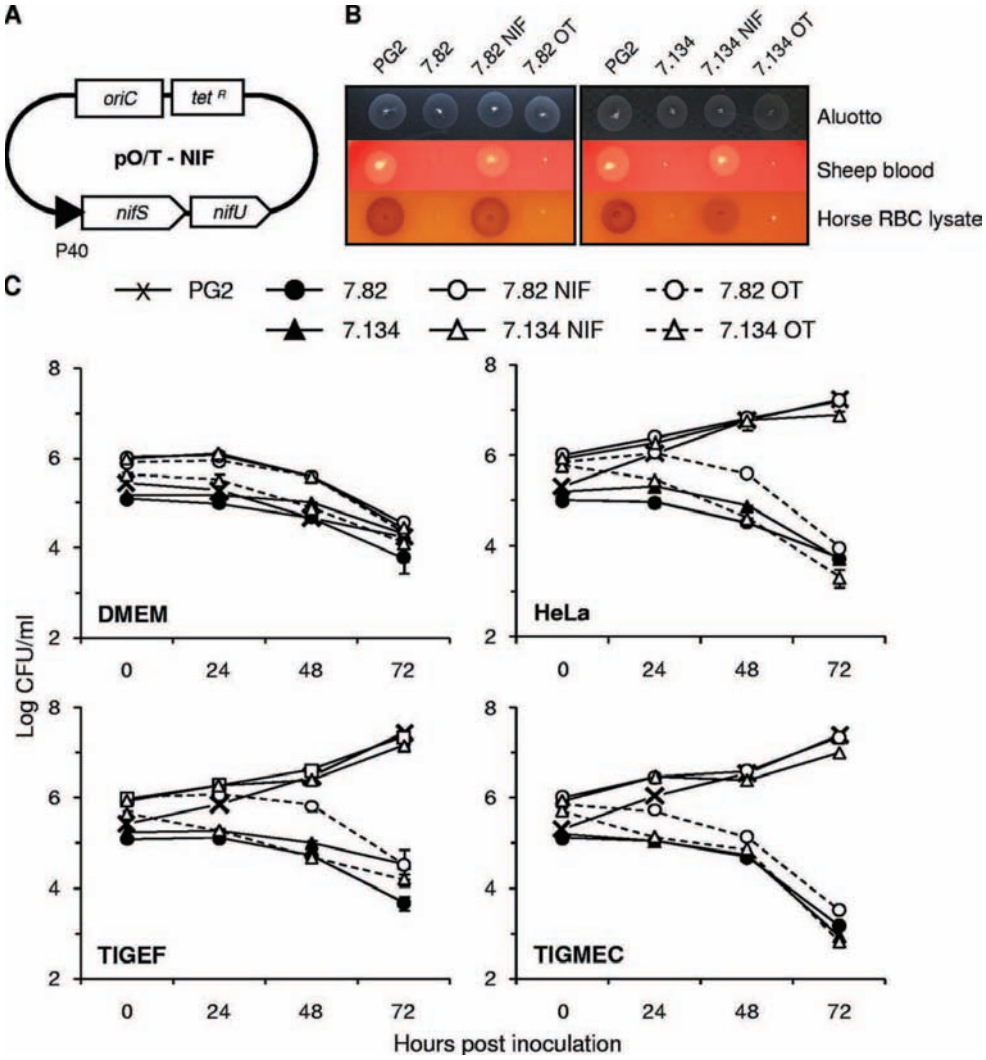


FIG. 5. Complementation of *M. agalactiae* NIF mutants. Schematic representation of the plasmid pO/T-NIF used for complementation studies (A). The NIF locus was cloned under the control of the P40 protein promoter region. *M. agalactiae* parental strain PG2, mutants T07.082 (7.82) and T07.134 (7.134), and mutants transformed with plasmid pO/T-NIF (7.82 NIF and 7.134 NIF) or the control plasmid p21-1miniO/T (7.82 OT and 7.134 OT) were assessed for colony development on Aluotto, 10% horse red blood cell (RBC) lysates, or 5% sheep blood agar plates (B) and for survival over a 72-h incubation in DMEM-based medium (DMEM) or in coculture with HeLa, goat fibroblast (TIGEF), or goat epithelial (TIGMEC) cells (C). The data are the means of at least three independent assays. Standard deviations are indicated by error bars. Serial dilutions of mycoplasma stocks were spotted on blood agar plates, and colony development was observed after 4 to 6 days of incubation at 37°C.

it is worth noting that best alignments for ICEA products of the PG2 strain were consistently obtained with the ICEC counterparts of *M. capricolum* subsp. *capricolum* (45), a member of the mycoides cluster which causes similar symptoms. Defining whether the growth deficiency phenotype observed in selected mutants resulted from the single transposon insertion or from phase variation or spontaneous mutation of other unrelated genes is essential, especially if regions apparently deprived of CDSs are involved. However, this is hampered by the difficulties in the genetic manipulation of these organisms. These difficulties have been overcome so far by complementation with the two mutants that had the most extreme phenotype.

Complementation studies confirmed that key functions conditioning *M. agalactiae* survival and proliferation under cell culture conditions were encoded by the NIF locus. In *M. agalactiae*, the locus is composed of two CDSs encoding homologues of nitrogen fixation proteins, NifS and NifU, two proteins involved in iron-sulfur [Fe-S] cluster biosynthesis. Documented in various organisms (14, 26), [Fe-S] cluster assembly systems are poorly understood in Gram-positive bacteria. Recent studies with *Enterococcus faecalis* identified the SUF machinery as the only [Fe-S] cluster biosynthetic system present in the *Firmicutes* genome (38). As expected by the taxonomic position of mycoplasmas, sequence features of the SUF machinery were identified in *M. agalactiae* NifS and NifU proteins. These include the amino acid sequence RSG IFCA surrounding Cys343 of MAG0720 (NifS), which is indicative of a group II (SUF-type) bacterial cysteine desulfurase, whose consensus sequence is RXGHHCA; this sequence clearly distinguishes NifS from group I enzymes of the iron-sulfur cluster, ISC (IscS-type), that display the sequence signature SSGSAC(T/S)S. Similarly, the MAG0730 (NifU) prod-

uct and SufU scaffold proteins share several features that distinguish them from IscU homologues. They both lack the LPPVK motif present in IscU and contain an 18- to 21-amino-acid insertion between the second and the third conserved cysteine residues. However, despite important sequence homologies with bacterial SUF machineries, mycoplasma NIF proteins exhibit several unique features.

The NIF locus present in mycoplasmas is a simplified version of more complex SUF operons and may encode cysteine desulfurases and scaffold proteins with unique biochemical properties. Its strict conservation among all mycoplasma genomes sequenced so far emphasizes its biological importance in mollicutes. Recent studies with a number of pathogenic bacteria, including *Mycobacterium tuberculosis*, *Shigella flexneri*, and the plant pathogen *Erwinia chrysanthemi*, have established a link between [Fe-S] cluster biosynthesis and virulence (24, 32, 39, 42). The central role played by bacterial SUF machineries in resistance to iron limitation and oxidative stress suggest that the NIF locus might play a similar role in *M. agalactiae*-host interactions. However, preliminary *in vitro* studies with *M. agalactiae* failed to reveal a particular susceptibility of NIF mutants when bacteria were exposed to oxidative stress or iron limitation. *In vivo* studies are in progress to determine the virulence of the NIF mutants in the animal host. The potential implication of this locus in a broad number of processes involving [Fe-S] proteins considerably increases the functions that can be affected in the NIF mutants. The additional functions that have been attributed to cysteine desulfurases (30), such as the biosynthesis of selenoproteins and multiple cofactors (biotin, lipoic acid, molybdopterin, thiamine, and NAD) as well as iron homeostasis and tRNA modifications, make the situation even more complex.

The growth-deficient phenotype exhibited by the NIF mutants under cell culture conditions and also potentially by other mutants (Fig. 2) revealed the decisive role played by metabolic functions in the adaptation of *M. agalactiae* to changing environments. A link between carbon metabolism and pathogenicity in mycoplasmas has already been suggested by several groups (5, 21–23, 36). The availability of a carbon source *in vivo* and its influence on bacterial pathogenicity has reignited interest in using carbon metabolic pathways as viable targets for antibiotic development (7). This might be particularly important for mycoplasmas, which have limited metabolic capacities and are dependent on the host for many nutrients (37).

Attempts to define the minimal amount of genetic information in various mycoplasma species revealed several discrepancies since orthologous genes found to be essential in one organism may be dispensable in another. This is illustrated here by the NIF locus, which was essential for axenic growth of *M. genitalium* (18, 25) but apparently dispensable in other mycoplasma species such as *M. pneumoniae*, *M. pulmonis*, and *M. agalactiae* (15, 18, 25). A number of situations may account for the occurrence of transposition events in essential genes. The identification of a paralog in *M. pulmonis* of the cysteine desulfurase-encoding gene provided a simple explanation for the apparent dispensability of the NIF locus in this species (15). Given the central role played by [Fe-S] proteins in a variety of fundamental biological processes, the absence of a paralog in the *M. agalactiae* genome suggests that essential functions might be performed by unrelated or very distantly related nonorthologous proteins.

The understanding of the basic molecular mechanisms underlying cellular life is of broad interest, and considerable effort has been devoted to establishing candidate minimal genomes. Our study provides a means for addressing this issue at a higher level of complexity, the host-cell context, and new opportunities to decipher mycoplasma-host interaction and virulence.

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Genome-Scale Analysis of *Mycoplasma agalactiae* Loci Involved in Interaction with Host Cells

Agnès Skapski[1,2], Marie-Claude Hygonenq[1,2], Eveline Sagné[1,2], Sébastien Guiral[1,2¤], Christine Citti[1,2], Eric Baranowski[1,2]*

1 INRA, UMR1225, IHAP, Toulouse, France, **2** Université de Toulouse, INP, ENVT, UMR1225, IHAP, Toulouse, France

Abstract

Mycoplasma agalactiae is an important pathogen of small ruminants, in which it causes contagious agalactia. It belongs to a large group of "minimal bacteria" with a small genome and reduced metabolic capacities that are dependent on their host for nutrients. Mycoplasma survival thus relies on intimate contact with host cells, but little is known about the factors involved in these interactions or in the more general infectious process. To address this issue, an assay based on goat epithelial and fibroblastic cells was used to screen a *M. agalactiae* knockout mutant library. Mutants with reduced growth capacities in cell culture were selected and 62 genomic loci were identified as contributing to this phenotype. As expected for minimal bacteria, "transport and metabolism" was the functional category most commonly implicated in this phenotype, but 50% of the selected mutants were disrupted in coding sequences (CDSs) with unknown functions, with surface lipoproteins being most commonly represented in this category. Since mycoplasmas lack a cell wall, lipoproteins are likely to be important in interactions with the host. A few intergenic regions were also identified that may act as regulatory sequences under co-culture conditions. Interestingly, some mutants mapped to gene clusters that are highly conserved across mycoplasma species but located in different positions. One of these clusters was found in a transcriptionally active region of the *M. agalactiae* chromosome, downstream of a cryptic promoter. A possible scenario for the evolution of these loci is discussed. Finally, several CDSs identified here are conserved in other important pathogenic mycoplasmas, and some were involved in horizontal gene transfer with phylogenetically distant species. These results provide a basis for further deciphering functions mediating mycoplasma-host interactions.

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* E-mail: e.baranowski@envt.fr

¤ Current address: Institut de Biologie et Chimie des Protéines,CNRS, Université de Lyon, Lyon, France

Introduction

The term "mycoplasma" is used trivially to describe bacteria belonging to the class *Mollicutes*, which includes the genus *Mycoplama*, as well as several other related genera [1]. These micro-organisms have evolved from a low G+C content Gram positive ancestor by "regressive evolution", resulting in massive genome reduction [2,3]. As a result, contemporary mycoplasmas lack a cell-wall and are commonly described as the smallest self-replicating organisms, because of the small size of their genome (580 to 1,400 kbp) and the paucity of their metabolic pathways. Mycoplasmas and ureaplasmas live in close contact with animal tissues, probably because of their limited metabolic capacity, a feature that is likely to have increased their dependence on hosts for a number of nutrients [2]. Mycoplasmas occur widely in nature and, despite their apparent simplicity, several species are successful pathogens of animals, in which they establish persistent infections and cause chronic disease [4].

Mycoplasma agalactiae is an important pathogen of small ruminants that causes contagious agalactia (CA), resulting in significant losses in the sheep and goat milk industries [5]. It is classified by the World Organization for Animal Health (OIE) as a notifiable disease and the clinical signs include mastitis, arthritis and kerato-conjunctivitis [5]. Contagious agalactia is also caused by several members of the mycoides cluster, including *M. mycoides* subspecies *mycoides* Large Colony type and *M. capricolum* subspecies *capricolum* [5]. Interestingly, while these mycoplasmas are phylogenetically distant from *M. agalactiae*, detailed *in silico* genomic analyses have revealed that extensive horizontal gene transfer has occurred between *M. agalactiae* and members of the mycoides cluster, and as a result these mycoplasmas may share a number of common cell surface functional domains [3,6]. Phylogenetically, *M. agalactiae* is closely related to *M. bovis* [7], a pathogen of large ruminants that causes clinical signs similar to those of contagious agalactia [8]. For all these ruminant mycoplasmoses, the factors involved in colonization, dissemination and pathogenicity are poorly understood. As a number of genetic tools and genomic data are available for *M. agalactiae* [6,9,10], this species is a useful model for studying the molecular players involved in infectious processes and thus furthering comprehension of pathogenic mechanisms in other mycoplasmas.

A common approach used to identify virulence genes in pathogenic bacteria is based on random transposon mutagenesis [11–13]. In mycoplasmas, this approach has mainly been applied

to study the minimal set of essential genes [14–16], but has also been successfully employed in a few cases to identify genes potentially involved in pathogenicity [17], gliding motility and adherence [18,19]. Such an approach is needed to further understanding of *M. agalactiae*, as *in silico* analyses of currently available ruminant mycoplasma genomes has failed to reveal unambiguously loci that might contribute to infection. Indeed, predicted *M. agalactiae* gene products have little to no similarity to virulence factors known in other bacteria, and 40% of the coding sequences (CDSs) have been annotated as hypothetical proteins with unknown functions [6]. For *M. agalactiae* and other ruminant species, one limitation of global transposon mutagenesis to identify virulence genes is the absence of a small laboratory animal model of infection, as *in vivo* screening in the natural ruminant hosts is constrained by both technical and ethical problems. To overcome these issues, we developed a method for high-throughput screening of *M. agalactiae* knockout mutants by co-cultivating *M. agalactiae* mutants with HeLa cells [20]. This assay allowed the selection of a number of genomic regions potentially required for growth in HeLa cell cultures, but dispensable in axenic conditions. Human epithelial surfaces are not a natural environment for *M. agalactiae*, so we extended our functional genomic study by using two caprine cell lines that are more relevant to the natural host context: goat mammary epithelial cells, TIGMEC, which are likely to be good targets based on the predilection of *M. agalactiae* for the mammary gland, and goat embryo fibroblasts, TIGEF.

Over 2000 *M. agalactiae* mutants were co-cultured with caprine cells and those showing a significant reduction in their capacity to grow were examined in detail, revealing 62 loci potentially required for propagation in the host environment. The relevance of these loci and the potential role of the genes at these loci in *M. agalactiae*-host interactions were analyzed.

Materials and Methods

Bacteria, cell lines and culture conditions

M. agalactiae reference strain PG2 (Refseq NC_009497) [6] was grown in Aluotto or SP4 medium as described previously [20]. Titers were determined by serial dilution in Dulbecco's phosphate-buffered saline (Invitrogen) containing 1% heat-inactivated horse serum (Invitrogen). *E. coli* DH10B (Invitrogen) was used for DNA cloning and plasmid propagation. The human and caprine cell lines used in this study included HeLa cells (ATCC CCL2), SV40 large T-antigen immortalized goat embryo fibroblasts (TIGEF) and similarly immortalized goat mammary epithelial cells (TIGMEC). TIGMEC were derived from milk epithelial cells [21]. Immortalized cells exhibited morphological and phenotypic features of parental milk epithelial cells and expressed cytokeratin, a specific marker of epithelial cells. Immortalized goat embryo fibroblasts were generated from carpal synovial membrane explants and displayed morphological features of fibroblastic cells [22]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM)-based medium, as described previously [20], composed of DMEM (high glucose, sodium pyruvate, and GlutaMAX-I; Invitrogen) supplemented with non-essential amino acids (NEAA; Invitrogen) and 10% heat-inactivated fetal calf serum (FCS; Eurobio).

M. agalactiae knockout mutant library

The library of knockout mutants was produced in *M. agalactiae* reference strain PG2 [20]. Transposon mutagenesis was carried out using plasmid pMT85, which does not replicate in mycoplasmas, but contains a modified version of transposon Tn*4001* (mini-Tn) conferring gentamicin resistance [23]. Mutants were collected from individual transformations to produce a representative library of 2,175 individual mutants. The pMT85-based library was propagated in SP4 medium supplemented with 500 µg cephalexin/ml (Virbac) and 50 µg gentamicin/ml (Invitrogen). Transposon insertion sites in the *M. agalactiae* chromosome were mapped by direct sequencing of the junction between the *M. agalactiae* genomic DNA and the 3′ end of the transposon using BigDye Terminator chemistry and oligonucleotide primer SG8 (Table 1). Direct sequencing was performed at the sequencing facility at the Bio-Medical Research Federative Institute of Toulouse (Toulouse, France). The 3′ end of the mini-Tn was defined using the orientation of the gentamicin resistance gene as the reference [23]. The Pip, MucB and P40 minus mutants were identified by PCR screening of the mutant library using specific oligonucleotide primers [20]. The mutants NifS1 and NifS2 have been described previously as mutants 7.82 and 7.134, respectively [20]. These mutants had a mini-Tn insertion at genomic positions 86804 (Pip), 88958 (MucB), 281483 (P40 minus), 87172 (NifS1) or 88125 (NifS2). The mini-Tn gentamicin resistance gene in the NifS1, Pip, MucB and P40 minus mutants was in the opposite orientation compared to the disrupted CDS, but in the same orientation in the NifS2 mutant.

High-throughput screening of *M. agalactiae* knockout mutant library in cell culture

A cell-culture assay was used to screen the *M. agalactiae* knockout mutant library and identify mutants displaying a growth-deficient phenotype [20]. Briefly, cells seeded in 96-well plates were inoculated with cultures of individual mutants using a 96-pin replicator (Boekel Scientific). Growth-deficient mutants were selected based on the titers reached at the end of the co-cultivation period. After one freeze-thaw cycle (−80°C/+37°C), co-cultures were spotted onto solid medium using a 96-pin replicator. The development of mycoplasma colonies was used as a cut-off point (Table 2). Culture stocks (inoculum) of *M. agalactiae* knockout mutants were tested by direct spotting onto solid medium. The titer reached by the wild-type after co-cultivation with HeLa cells [20], TIGMEC or TIGEF cells was not influenced by variations in the initial inoculum size (data not shown). *M. agalactiae* was unable to proliferate in cell-culture medium alone [20].

RNA extraction, RT-PCR amplification and primer extension

Total RNA was extracted from 48 hour cultures of *M. agalactiae* using the TRIzol method (Invitrogen). RNA samples were stored at −80°C. The RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm and by agarose gel electrophoresis. RT-PCRs were carried out with the Access RT-PCR System kit (Promega), using 1 µg of total RNA treated with DNAse (RNase-free DNAse, Promega). Reactions were incubated at 45°C for 45 min, 94°C for 2 min, then through 30 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 30 sec, with a final extension incubation of 7 min at 72°C. Amplification products were analyzed by agarose gel electrophoresis. Primer extension was carried out using 15 µg of total RNA, 2 pmol of the labeled oligonucleotide primer 5′-6FAM-NifS1 (Table 1), and 200 units of Superscript III Reverse Transcriptase (Invitrogen). RNAs were denatured by 5 min incubation at 65°C. Reverse transcription reactions were performed at 42°C for 50 min, followed by enzyme inactivation at 75°C for 15 min. The cDNAs were treated with RNAse A (Promega) for 30 min at 37°C, ethanol precipitated and resuspended in 10 µl formamide and 0.4 µl of GS-400HD ROX size standards (Applied Biosystems). Product sizes were

Table 1. Oligonucleotides used in this study.

Name	Sequence (5′→3′)
SG5	TTTTACACAATTATACGGACTTTATC
SG8	GAGTCAGTGAGCGAGGAAGC
P40_RF_CC	ACGGGGCTAAAGAAGCTGAT
CCP40-03	TGGTTATATTTTCCATATCTTTC
Pip-P40_F	GCAATTGAGAATTTTATTAAAGG ATAAATA–ATGAAAACAAATAGA AAAATATTGTTTGGT
Pip-P40_R	ACCAAACAATATTTTTCTATTT GTTTTCAT–TATTTATCCTTTA ATAAAATTCTCAATTGC
Pip-86804_F	GCCAGCCATATGGTGCATATTTAG
Pip_F	TATTCGACCAAAGAGGGTGT
Pip_R	TCATCAAAATCACCACCAAG
NifS_F1	TCAGCCGACATTATTCATGG
NifS_R1	CACCGGCTTTTAATTTTTGC
NifS_F2	TGTCACAAAGTTGGAGCAAT
NifS_R2	ACGAAGGAATCGTTACAAGC
NifU_F	AGGGTTTCGCTAGGGGTTTA
NifU_R	CTGTGCGCGCTTACAAAGTA
5′-6FAM-NifS1[a]	TAGTTCTTGTGCTAACCGAATA

[a]Oligonucleotide labeled by a 6-carboxyfluorescein (FAM) 5′-modification.
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analyzed using the ABI3730 sequencer (Applied BioSystems) at the GenoToul genomic platform of Toulouse (France).

A reporter system for the detection of transcriptional promoter sites in *M. agalactiae*

The surface antigen P40 CDS MAG2410 was used as a reporter gene to assess transcriptional promoter activity in *M. agalactiae*. DNA sequences to be tested were cloned upstream of CDS MAG2410 using the plasmid p20-1miniO/T as a vector [20]. The 3′ end of the *pip* gene was amplified by PCR using the primers Pip-86804_F and Pip-P40_R (Table 1) to generate a 191 bp fragment overlapping CDS MAG2410. CDS MAG2410 was amplified using Pip-P40_F and CCP40-03 (Table 1) to generate a 1188 bp fragment overlapping the 3′ end of the *pip* gene. The two overlapping fragments were assembled by PCR amplification using the primers Pip-86804_F and CCP40-03 (Table 1). The resulting PCR products were cloned into the pGEM-T Easy vector (Promega), before sub-cloning into the *Not*I site of plasmid p20-1miniO/T. PCR amplifications were performed using the proofreading Phusion High Fidelity polymerase (Finnzymes). Before cloning into pGEM-T Easy, 3′-terminal deoxyadenosine residues were added to blunt-ended PCR products by following the A-tailing procedure provided by the supplier (Promega). Cloned sequences were verified by DNA sequencing. CDS MAG2410 alone or with its own promoter sequence was used as negative and positive controls, respectively. PCR amplifications were performed using primers P40_RF_CC and CCP40-03 (positive control), and Pip-P40_F and CCP40-03 (negative control)(Table 1).

A surface antigen P40-knockout mutant (P40 minus; see above) was used to test the expression of P40 from the different plasmid constructs. *M. agalactiae* cells (10^8 to 10^9 CFU) were transformed by electroporation using 1 to 3 μg of plasmid DNA, as described previously [20]. After 3 hours incubation in non-selective medium, cells were allowed to grow in the presence of appropriate antibiotic for 24 hours before plating on selective solid medium. Transformants were picked after 4 to 7 days and subcultured in selective SP4 medium. The expression of *M. agalactiae* surface antigen P40 was tested by Western blotting.

Table 2. High-throughput detection of *M. agalactiae* mutants unable to grow on cultured cells.

Cut-off value (CFU titers)[a]	TIGMEC[b]	TIGEF	HeLa
0 (10^4 CFU/ml)	15 (0.7%)[c]	15 (0.7%)	25 (1.1%)
10 (10^5 CFU/ml)	26 (1.2%)	96 (4.4%)	153 (7.0%)

[a]The cut-off value is the number of colonies counted on solid medium following 3 days co-cultivation of *M. agalactiae* knockout mutants with cells. Titers in parentheses indicate the predicted titers in the co-culture.
[b]Number of *M. agalactiae* growth deficient mutants selected on goat mammary epithelial cells (TIGMEC), goat embryonic fibroblast cells (TIGEF), and HeLa cells (HeLa).
[c]Percentage of growth deficient mutants selected from the mutant library.
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Western blotting and immunodetection of *M. agalactiae* lipoprotein P40

Mycoplasmas grown in SP4 medium were collected by centrifugation at 10,000× g and resuspended in Dulbecco's phosphate-buffered saline (Invitrogen). The protein concentration was determined using the Quick Start Bradford protein assay (Bio-Rad). For Western blotting, total proteins (0.5 μg) were separated by SDS-PAGE using the Mini-Protean II electrophoresis system (Bio-Rad) and transferred to Protran nitrocellulose membranes (Whatman). Membranes were blocked in Tris-buffered saline (TBS) (10 mM TrisHCl, pH 7.4; 150 mM NaCl) containing 5% skim milk for 2 hours, then incubated overnight at 4°C with a sheep anti-P40 serum at a dilution of 1/500 in TBS containing 0.05% Tween 20 and 10% heat-inactivated horse serum (Invitrogen). Western blots were developed using horseradish peroxidase conjugated secondary antibody raised in rabbits (DAKO) and 4-chloro-naphthol as substrate. Sheep serum raised against the *M. agalactiae* surface antigen P80 was used as a control (dilution of 1/200). The anti-P40 and anti-P80 sheep sera were produced by animal immunization with P40 or P80 recombinant proteins, respectively (data not shown).

Results and Discussion

High-throughput identification of *M. agalactiae* growth-deficient mutants upon co-cultivation with host cells

Our group has previously reported the construction of a library of *M. agalactiae* knockout mutants together with the development of a high-throughput screening method based on the co-cultivation of *M. agalactiae* mutants with HeLa cells [20]. Since human epithelial cells are not a natural environment for *M. agalactiae*, we subsequently screened the library with two caprine cell lines, which are likely to be more relevant to the normal host-cell interactions involved in infections with this pathogen. The goat mammary epithelial cells, TIGMEC, are likely to be particularly relevant given the predilection of *M. agalactiae* for the mammary gland. HeLa cells were used as a control.

The ability of individual mutants to grow in the presence of these three cell lines was assessed after 3 days of co-cultivation by

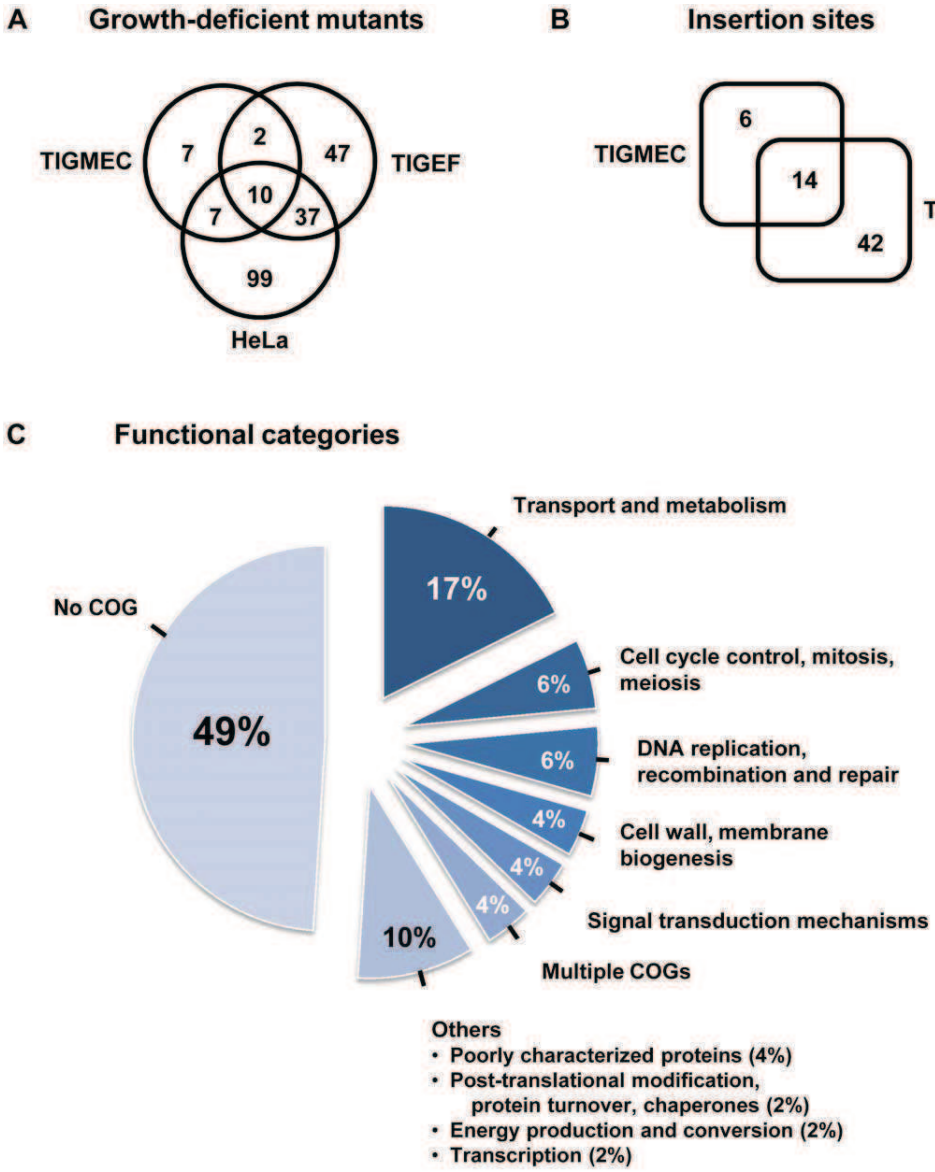

Figure 1. Overall distribution of *M. agalactiae* growth deficient mutants selected after co-cultivation with host cells. (A) Number of mutants selected on goat mammary epithelial cells (TIGMEC), goat embryo fibroblasts (TIGEF) and/or HeLa cells. (B) Number of unique transposon insertion sites identified in mutants selected for their inability to grow on TIGMEC and/or TIGEF. Some mutants had the same insertion site. (C) Distribution in COG categories of the 46 CDSs found disrupted in growth deficient mutants selected on caprine cells (see panel B) [38]. Multiple COG proteins include MAG1490 and MAG2120, which fall into several COG categories (energy production and conversion, C-COG, coenzyme transport and metabolism, H-COG and general function prediction only, R-COG for MAG1490; general function prediction only, R-COG, signal transduction mechanisms, T-COG, transcription, K-COG and replication, recombination and repair, L-COG, for MAG2120).
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directly spotting the cultures onto solid medium and comparing the titers (see Materials and Methods). A total of 209 growth-deficient mutants were selected out of 2,175 tested, using a cut-off value of 10 colonies per plate, which corresponded to a titer of 10^5 CFU/ml in co-cultures (Table 2). The mutants unable to grow in each cell line are shown in Fig. 1A, with a detailed description of those unable to grow on caprine cells provided in Tables 3 and 4. Some differences were seen between the repertoire of mutants identified as growth deficient on HeLa cells in the current study and those identified in our previous study [20], with only 61% common to both studies. This reflects the limitations of these assays, including some cross contamination between individual mutants stored in 96-well plates. These were found to hamper the selection of mutants with reduced growth capacities in cell culture (data not shown) and to affect the reproducibility of the screening from one experiment to the other.

Some mutants (27%) displayed reduced growth capacities on multiple cell types, while the majority (73%) exhibited this phenotype on one cell line (Fig. 1A). Interestingly, only a small number of mutants were selected on TIGMEC, compared to the number selected on TIGEF and HeLa cells (Table 2). It is possible that the tolerance of *M. agalactiae* to transposon mutagenesis increases when grown on a cell type similar to its natural environment, but this hypothesis needs to be confirmed with a larger panel of host cell types.

Table 3. *M. agalactiae* CDSs identified by high-throughput screening for their reduced growth capacities on cultured cells.

CDS name[a]	Cells[b]	No. of mutants (no. of insertion sites)[c]	% CDS (orientation)[d]	Gene	Gene product (COG)[e]	Predicted Localization[f]	Detected by MS/MS[g]
MAG0490	F	1 (1)	0.62 (−)		CHP	M	−
MAG0640	F	1 (1)	0.71 (−)	*asnA*	Aspartate-ammonia ligase (E)	C	−
MAG0720	E, F *	2 (2)	0.18 (−)/1.00 (+)	*nifS*	Cysteine desulfurase (E)	C	−
MAG0890	E, F *	2 (1)	0.85 (+)	*hprK*	Hpr kinase phosphorylase (T)	C	+
MAG1180	E, F *	1 (1)	0.95 (−)	*pepP*	XAA-PRO aminopeptidase (E)	C	+
MAG1330	F *	1 (1)	0.31 (−)		CHP DUF285 family, predicted lipoprotein	M	−
MAG1430	E, F *	6 (3)	0.15 (+)/0.84 (+)/ 0.85 − 0.95 (−)		HP	M	+
MAG1490	F *	1 (1)	0.86 (−)	*ldhD*	D-lactate dehydrogenase (CHR)	IM	+
MAG1500	F	2 (1)	0.56 (+)		Esterase lipase (R)	C	−
MAG1540	F *	1 (1)	0.27 (−)	*tig*	Trigger factor (O)	C	+
MAG1740	F	1 (1)	0.32 (−)	*gidA*	Glucose-inhibited division protein A (D)	C	+
MAG1860	F *	1 (1)	0.63 (−)	*gidB*	Methyltransferase GidB (M)	C	−
MAG1890	F	1 (1)	0.11 (−)		HP	M	+
MAG2110	E	1 (1)	0.08 (+)		Protein phosphatase (T)	C	−
MAG2120	E, F *	1 (1)	0.53 (−)	*pknB*	Serine/threonine-protein kinase (RTKL)	M	+
MAG2540	F *	20 (1)	0.57 (−)		HP, Vpma-like, predicted lipoprotein	M	+
MAG2680	F *	1 (1)	0.60–0.70 (−)		HP	M	+
MAG2870	F *	2 (2)	0.16 (−)/0.72 (+)		CHP, predicted lipoprotein	M	−
MAG2930	E *	1 (1)	0.01 (−)	*atpA*	ATP synthase á chain (C)	IM	+
MAG2960	F *	1 (1)	0.17 (−)		CHP, predicted lipoprotein	M	−
MAG3030	F	3 (1)	0.31 (+)		HP	M	−
MAG3350	F *	1 (1)	0.18 (−)		HP	M	−
MAG3370	F	1 (1)	0.72 (−)		CHP	M	−
MAG3480	F *	1 (1)	0.70 (−)		HP	C	+
MAG3720	E, F	1 (1)	0.10 (+)		CHP	IM	−
MAG3740	F *	1 (1)	0.2–0.4 (+)	*mraZ*	MraZ (S)	IM	−
MAG3790	E, F *	10 (1)	0.95 (−)	*uvrA*	UvrABC system protein A (L)	C	+
MAG3860	F *	1 (1)	ND (−)		CHP	IM	−
MAG4200	F	1 (1)	0.97 (+)		CHP	C	−
MAG4380	F *	3 (2)	0.50–0.75 (−)/ 0.88 (+)		P115-like ABC transporter ATP binding protein (D)	C	+
MAG4650	E	1 (1)	0.98 (−)		Phosphomannomutase (G)	C	+
MAG4740	F	1 (1)	0.11 (+)		HP, predicted lipoprotein	M	+
MAG4820	E	2 (1)	0.97 (−)		CHP (M)	C	−
MAG4950	E, F *	1 (1)	0.79 (−)		HP, predicted lipoprotein	M	−
MAG5000	F	4 (1)	0.22 (+)		HP	M	−
MAG5150	F *	1 (1)	0.91 (−)		HP, predicted lipoprotein	M	+
MAG5910	F	1 (1)	0.72 (+)		5′ nucleotidase, predicted lipoprotein (F)	M	+
MAG6090	F	1 (1)	0.96 (−)		HP, predicted lipoprotein	M	−
MAG6450	E	1 (1)	0.87 (+)		CHP	C	−
MAG6690	F	1 (1)	0.67 (−)		HP	M	−
MAG6760	F	1 (1)	0.07 (−)	*chrA*	Chromate transport protein (P)	M	−
MAG6770	F *	1 (1)	0.99 (−)	*chrA*	Chromate transport protein (P)	M	+
MAG6870	F	2 (2)	0.60 (−)/0.70 (−)	*dnaX*	DNA polymerase III subunits gamma and tau (L)	C	+
MAG6960	E, F *	1 (1)	0.58 (−)	*apt*	Adenine phosphorybosyltransferase (F)	C	+

Table 3. Cont.

CDS name[a]	Cells[b]	No. of mutants (no. of insertion sites)[c]	% CDS (orientation)[d]	Gene	Gene product (COG)[e]	Predicted Localization[f]	Detected by MS/MS[g]
MAG7100	F	1 (1)	0.56 (−)	*vpmaZ*	Variable surface lipoprotein D (VpmaZ precursor)	M	+
MAG7200	F	1 (1)	0.74 (+)	*scpB*	Segregation and condensation protein B (K)	C	+

[a]CDS found disrupted in *M. agalactiae* growth-deficient mutants [6].
[b]Letters E and F indicate that the *M. agalactiae* growth-deficient mutants were selected on TIGMEC or TIGEF cells, respectively. Asterisks (*) indicate mutants that were also selected during high-throughput screening on HeLa cells.
[c]For each CDS, the number of mutants identified during the screening on caprine cell lines is indicated, as well as the number of different mini-Tn insertion sites.
[d]For each CDS, the relative position and the orientation of the inserted transposon are indicated. Mini-Tn insertion sites were determined by direct sequencing of genomic DNA, and their positions were defined based on the published genome sequence (NC_009497).
[e]Hypothetical proteins (HP) have no homolog outside the species *M. agalactiae*. Conserved hypothetical proteins (CHP) share sequence similarity with proteins of unknown function identified in other *Mollicutes* or other bacteria. COG categories of encoded proteins are indicated in parentheses [38].
[f]Protein localization was predicted using TMHMM [41,42]; membrane (M), cytosolic (C), or indirectly linked to the membrane (IM).
[g]Proteins with peptides detected during proteomic analysis of gene products expressed by *M. agalactiae* strain PG2 in axenic culture are identified by a plus sign (+), while proteins not detected are identified by a minus sign (−) [32].
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Mapping of transposon insertion sites in the genome of growth-deficient *M. agalactiae* mutants

Direct genomic sequencing was performed to determine the position of the transposon in mutants found to be inhibited on caprine cell lines. The 110 mutants had 62 unique insertion sites that mapped within 46 different coding sequences (CDS) and 9 non-coding regions (NCR) (Fig. 1B and Tables 3, 4). The examination of DNA sequence chromatograms failed to reveal multiple transposon insertion events in the *M. agalactiae* chromosome (data not shown).

As shown in Figure 1C, most of the disrupted CDSs have been annotated as hypothetical proteins (HP) of unknown function (no COG). Within this category, predicted surface lipoproteins were highly represented 2.1 times more than would be expected [6]. Because of the absence of a cell wall in mycoplasmas, cell surface lipoproteins are thought to be key players in modulating interactions with the host. Interestingly, 20 mutants with a disruption in the same lipoprotein gene (MAG2540) were inhibited on the TIGEF, but not the TIGMEC (Table 3). All had the transposon inserted at the same position, suggesting that they were probably siblings derived from the same parental clone. The repeated selection of this particular mutant suggests a role for MAG2540 in the proliferation of *M. agalactiae* on TIGEF, even though it is dispensable in axenic culture medium and in the presence of TIGMEC. The function of this CDS is unknown, but it is predicted to encode a surface lipoprotein (Vpma-like lipoprotein) with similarities to some domains of the hypervariable Vpma lipoproteins of *M. agalactiae* [24]. Because MAG2540

Table 4. *M. agalactiae* NCRs identified by high-throughput screening with host cells.

Name[a]	Cells[b]	No. of mutants (no. of insertion sites)[c]	Size of NCR	Genomic position (orientation)[d]	Genetic environment of NCR[e]
NCR A	F	1 (1)	67 nt	31900 (+)	HP (MAG0310) and HP (MAG0320)
NCR B	E, F *	7 (1)	676 nt	388694 (+)	*ptsG* (MAG3250) and CHP DUF285 family (MAG3260)
NCR C	E, F *	2 (1)	740 nt	402664 (+)	HP (MAG3390) and CHP (MAG3400)
NCR D	F	1 (1)	351 nt	460191 (+)	Pseudogene of CHP (MAG3880) and pseudogene of CHP (MAG3890); vestige of ICEA
NCR E	F *	1 (1)	423 nt	469389 (+)	HP (MAG3950) and CHP (MAG3960); AMIGene CDS prediction of 52 AA (from 469474 to 469319); vestige of ICEA
NCR F	F *	2 (1)	330 nt	473081 (+)	HP (MAG4010) and HP (MAG4020); vestige of ICEA
NCR G	E	1 (1)	802 nt	648734 (−)	*gyrA* (MAG5630) and *hsdS* (MAG 5640); AMIGene CDS prediction of 67 AA (from 648894 to 648694)
NCR H	F	1 (1)	290 nt	761860 (−)	HP (MAG6430) and putative prophage protein ps3 (MAG6440)
NCR I	F *	2 (2)	80 nt	843595 (+)/843634 (+)	*cmk* (MAG7250) and HP (MAG7260)

[a]NCRs were labeled with a letter based on their position in the genome of *M. agalactiae*.
[b]Letters E and F indicate NCRs carrying insertions in mutants with reduced growth capacities on TIGMEC and/or TIGEF, respectively. Asterisks (*) indicate mutants that were also selected during high-throughput screening on HeLa cells.
[c]For each NCR, the number of mutants identified during the screening with caprine cells is indicated, as well as the number of insertion sites.
[d]Transposon insertion sites were determined by direct sequencing of genomic DNA and their positions were defined based on the published genome sequence (NC_009497). The orientation of the transposon is indicated in parentheses.
[e]Surrounding CDS and mini-Tn disrupted AMIGene CDS predictions; CDS names are given in parenthesis; vestige of ICEA indicates an NCR located within a 20 kb locus containing a vestige of an integrative conjugative element (ICEA).
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knockout mutants were also repeatedly selected on HeLa cells, it is possible that, in the absence of specific receptors on target cells such as the TIGMEC, this Vpma-like lipoprotein facilitates binding to ubiquitous structures on mammalian cell surfaces. This implies that growth of *M. agalactiae* in cell cultures requires a close interaction with the cells that is lost in the MAG2540 knockout mutants when growing on TIGEF or HeLa cells. As expected for bacteria with small genomes and reduced metabolic pathways, "transport and metabolism" (COG categories E, F, G, H and P) was the largest functional category involved in interactions between *M. agalactiae* and caprine cells, with 17% of the mutants selected having insertions in genes involved in these functions (Fig. 1C). Since the wild-type is unable to proliferate in cell-culture medium alone [20], it is most likely that these mutants are unable to transport or metabolize some of essential nutrients provided by the cultured cells. The remaining CDSs for which a predicted function had been assigned were distributed across a broad range of functional categories (Fig. 1C), often without any predictable correlation with their role in interactions with caprine cells.

Some of the loci identified in mutants selected on caprine cell lines (Tables 3 and 4), were also identified as being required for growth on HeLa cells, either in this study (Tables 3 and 4) or in our previous study [20]. This suggests that these loci may be involved in general processes mediating interactions between *M. agalactiae* and mammalian cells. The functions encoded by several of these, including DNA repair (*uvrA*), nucleotide metabolism (*apt*), iron-sulfur cluster biosynthesis (*nifS*), and protein folding (*tig*), contribute to stress tolerance and virulence in a number of pathogenic bacteria [25–31].

A global proteomic analysis of gene products expressed by *M. agalactiae* strain PG2 in axenic culture [32] detected approximately 50% of the CDSs found to be required for growth on caprine cells (Table 3). Whether this reflects the limited sensitivity of the proteomic approach, or differential expression by *M. agalactiae* under axenic and co-culture conditions is not known.

About 20% of the mutants selected on caprine cells had an insertion in a NCR (Table 4). The importance of NCRs in the biology of *M. agalactiae* remains largely unexplored, but several are likely to include transcriptional promoters and other regulatory regions. Re-examination of the 9 mutated NCRs using the AMIGene annotation tool [33] revealed that two, NCR E and G, had an insertion within short CDSs (Table 4) that had not been annotated previously but that could encode products with a role in host-cell interaction. Interestingly, transcriptional activity in NCRs has been reported for a number of mycoplasmas [34,35], suggesting an active role for these regions. NCRs D, E and F (Table 4) are part of a 20 kb locus containing a vestige of an integrative conjugative element (ICEA) [6,36]. The mechanism by which NCRs may regulate the proliferation of *M. agalactiae* in cell culture remains to be elucidated.

Finally, several CDSs required for growth on host cells are conserved in other ruminant mycoplasma species, and/or were involved in the massive horizontal gene transfer (HGT) that occurred between *M. agalactiae*, *M. bovis* and members of the phylogenetically distant mycoides cluster (Table S1). Two CDSs (MAG2870 and MAG6690) were also predicted to have undergone HGT between the avian mycoplasma species *M. synoviae* and *M. gallisepticum* (Table S1). About 70% of these CDSs involved in HGT encode membrane-associated proteins, and thus are likely to play a role in mycoplasma–host interactions. The genome-scale analysis of *M. agalactiae* in cell culture may assist in understanding pathogenic processes involved in other mycoplasma infections.

The frequent occurrence of promoter regions in the genome of *M. agalactiae* reduces polar effects mediated by integrated transposon sequences

As mentioned above, some CDSs required for growth on cell cultures fell into functional categories (Fig. 1C) that had no obvious correlation with mycoplasma-host interactions. This raised the question of whether growth-deficient phenotypes can result from a polar downstream effect rather than the effect on the gene disrupted by the transposon insertion. The compact mycoplasma genome, which is often organized into highly dense co-linear gene clusters with operon-like structures, suggests that this might be likely [3].

To evaluate the potential influence of the mini-Tn insertions on gene expression from these operon-like structures, we analyzed the 4 kb co-linear gene cluster *pip-nifS-nifU-mucB* (MAG0710 to MAG0740) (Fig. 2). This cluster was of particular interest because: (i) *nifS* has been shown to be essential for proliferation of *M. agalactiae* on cell cultures, and two knockout mutants, NifS1 and NifS2, were identified by high-throughput screening on caprine and human cells (Table 3); and (ii) these co-linear genes are likely to be co-transcribed by a promoter located upstream of *pip*, based on genome data indicating short intergenic distances (Fig. 2A). Indeed, the operon-like structure of the *pip-nifS-nifU-mucB* cluster was further supported by our transcriptional analyses, which detected overlapping transcripts by RT-PCR (Fig. 2A). Two mutants, Pip and MucB, with a transposon inserted into the corresponding genes, were searched for and found in the mutant library (see Materials and Methods), but both are able to grow on all three cell lines. When tested individually with TIGMEC, both mutants had the wild-type phenotype (Fig. 2B). This result was surprising, at least for the Pip mutant, which was expected to have a similar phenotype to that of the NifSs mutants because of the predicted polar effect of the transposon insertion (Fig. 2A).

RT-PCR amplification using oligonucleotide primers bracketing the mini-Tn integration site in mutants Pip, NifS1, and NifS2 did not detect mRNA (RT-PCR II in mutants Pip and NifS1, and RT-PCR IV in mutant NifS2), but, unexpectedly, transcripts were detected downstream of the mini-Tn (RT-PCR III and IV in mutants Pip and NifS1), suggesting that there may be a promoter within *pip* or the mini-Tn. The presence of a promoter within the integrated mini-Tn sequences was suggested by the identification of RNA transcripts that overlapped the 3′ end of the mini-Tn and *nifS* in Pip and NifS1 mutants (data not shown). The extension of transcription beyond the 3′ end of the mini-Tn was probably responsible for the expression of downstream genes in knockout mutants of *M. agalactiae* and may have influenced the orientation of the mini-Tn in the mutant library [20]. Although promoter sequences in the mini-Tn remain to be formally demonstrated, constitutive gene expression from the 3′ end of the mini-Tn can also be viewed as another outcome of transposon mutagenesis. Experiments are in progress to determine the role of this mechanism in mutants unable to grow in cell cultures that harbor a transposon within a NCR.

Recent studies on *Mycoplasma genitalium* have revealed that the genome of this human pathogen is actively transcribed, and contains multiple cryptic promoters [34]. This led us to further explore transcriptional activity within the *pip-nifS-nifU-mucB* cluster, and to examine the promoter activity at the 3′ end of the *pip* gene. A reporter system was developed to detect promoter sequences in *M. agalactiae* (Fig. 2C). Lipoprotein P40 (MAG2410) is a surface antigen of *M. agalactiae*, the expression of which can be detected using specific antibodies. The successful complementation of a *M. agalactiae* mutant, P40 minus, harboring a transposon

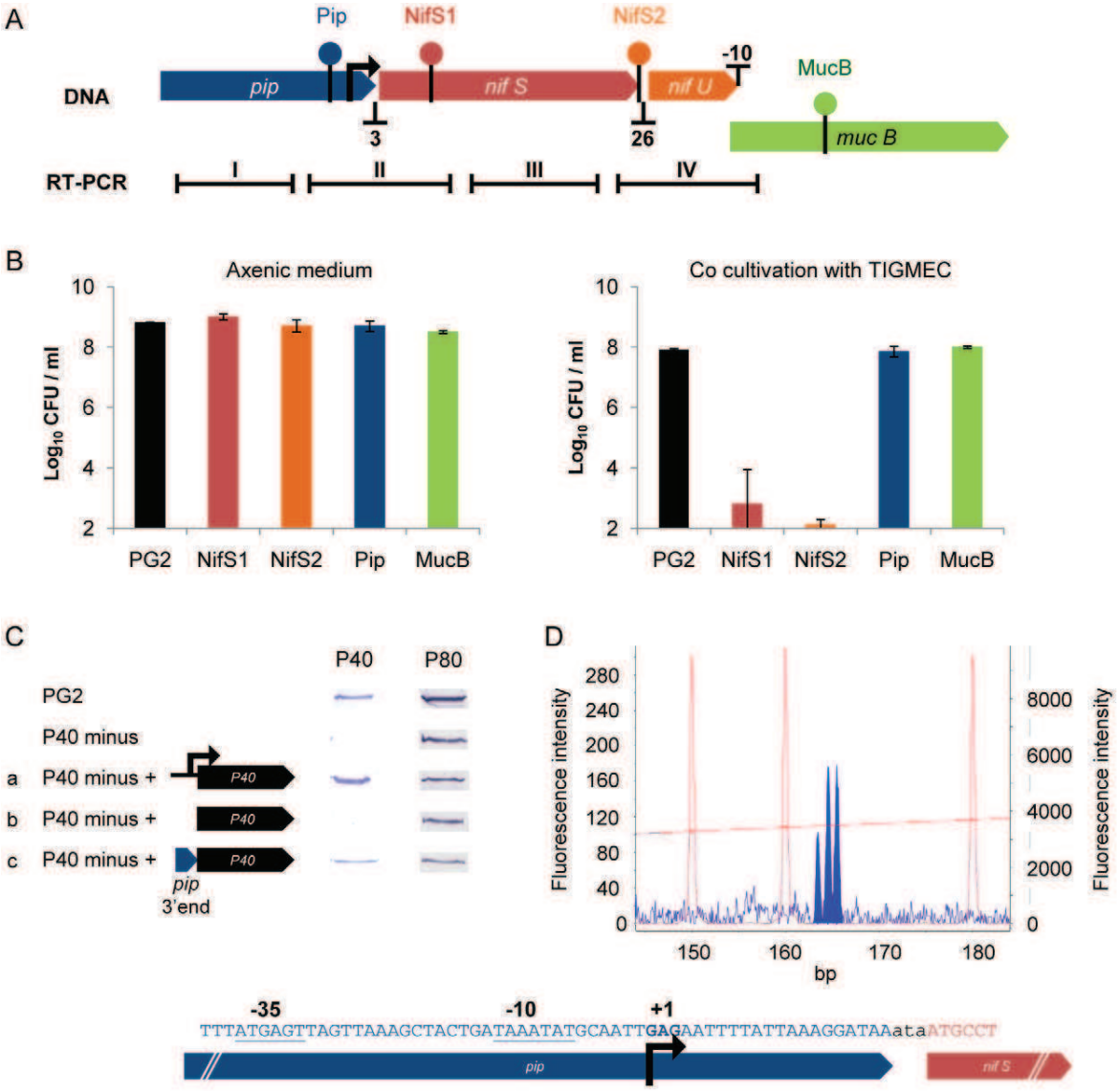

Figure 2. The *nif* locus in *M. agalactiae* is a promoter-less CDS cluster inserted within a transcriptionally active region. (A) Schematic representation of the 4 kbp co-linear gene cluster *pip-nifS-nifU-mucB* indicating the four regions amplified by RT-PCR. Intergenic distances in nucleotides are indicated below. Transposon insertion sites are indicated by filled circles using a different color code for each mutant. RNA regions amplified by RT-PCRs I to IV are indicated by black bars. The promoter identified in the 3′ end of *pip* is indicated by a black arrow. (B) Bar graph indicating the titers obtained, after cultivation in axenic media or in the presence of TIGMEC, of *M. agalactiae* strain PG2 or mutants NifS1, NifS2, Pip and MucB. Titers were determined after 48 h cultivation in axenic medium and after 72 h co-cultivation with TIGMEC. The bars indicate the means of three independent assays, with standard deviations indicated by error bars. (C) Identification of promoter sequences in *M. agalactiae* using a new reporter system. The surface antigen P40-knockout mutant (mutant P40 minus) was complemented using plasmid constructs containing the P40 coding sequence preceded by its own promoter (a), without a promoter (b), or preceded by the 3′ end of *pip* (c). The expression of the P40 antigen was detected by Western blotting. The surface antigen P80 was used as control. (D) Chromatogram obtained after primer extension of total RNA extracted from a *M. agalactiae* PG2 culture with primer 5′-6FAM-NifS1 (Table 1) as described in the Materials and Methods. Red peaks correspond to the GS-400HD ROX internal lane standards and the shaded blue peaks to the primer extension product. The size is indicated on the X-axis in base pairs. The peak height indicates the fluorescence intensity (arbitrary units) with GS-400HD ROX fluorescence measured on the right Y-axis and FAM fluorescence on the left Y-axis. The position of the transcriptional start point in CDS MAG0710 (*pip*) identified by primer extension experiments is indicated. Putative −10 and −35 regions are underlined. The transcriptional start points are indicated in bold.
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within the P40 lipoprotein gene, with a plasmid expressing P40, led us to develop a reporter system to detect promoter activity. Western blotting analyses confirmed that the 157 nucleotides at the 3′ end of the *pip* gene, when inserted at the 5′ end of the P40 lipoprotein gene, promote complementation of the P40 minus mutant.

Finally, the transcriptional start point at the 3′ end of the *pip* gene was mapped by primer extension using the oligonucleotide 5′-6FAM-NifS1 (Table 1). A major extension product with a size of between 163 and 165 nucleotides was obtained, indicating that the transcriptional start point was 31 nucleotides upstream of the stop codon of the *pip* gene (Fig. 2D). The detection of multiple

extension products differing in length by a single nucleotide is a common finding in mycoplasma promoters [34,37].

The genomes of mycoplasmas are characterized by a low G+C content. This particular feature may favor the occurrence of primary or secondary promoter regions in the genome of *M. agalactiae*, even within coding sequences such as this promoter within the 3′ end of the *pip* gene. The high density of promoters within the genome of *M. agalactiae* may have important evolutionary consequences, facilitating chromosomal rearrangements and gene shuffling in these rapidly evolving organisms.

Gene involved in the interaction of *M. agalactiae* with host cells may have been hitchhiking across evolution

Although polar effects resulting from the disruption of co-transcribed gene clusters cannot be ruled out, the identification of multiple transposon insertion sites in the same region suggests that important factors mediating the interaction between *M. agalactiae* and host cells may map to these loci. Among the 62 loci identified after screening with host cells, six loci mutants had insertions in the same CDS or NCR, but at different positions (Tables 3 and 4). CDSs with multiple insertion sites included two HP predicted to be exposed on the cell surface (MAG1430 and MAG2870), and cytosolic proteins with homologies with cysteine desulfurase (MAG0720), the P115-like ABC transporter ATP binding protein (MAG4380), and DNA polymerase III subunits gamma and tau (MAG6870). Only one NCR (NCR I) was found containing two different insertions that inhibited the capacity of *M. agalactiae* to grow on host cells. Interestingly, CDSs MAG0720 (*nifS*) and MAG6870 (*dnaX*) each belong to co-linear gene clusters (Fig. 3) that are highly conserved in *Mollicutes* (Fig. S1, S2, and S3). The remarkable conservation of these two gene clusters led us to examine the co-localization of the 46 CDS identified as being required for growth of *M. agalactiae* on cultured cells, and to compare the synteny of these CDSs using complete genome sequences of mycoplasmas available in public databases. A total of 8 highly conserved co-linear gene clusters were identified (Fig. 3 and Fig. S1, S2, and S3). Several of these clusters encode proteins predicted to be involved in specific functions, including [Fe-S] cluster biosynthesis (NIF), chromate transport (CHR), cell division (MRAZ) and DNA replication, recombination and repair (DNAX). The potential role of these CDS clusters in the interaction between *M. agalactiae* and host cells was further supported by the similar effect of transposon insertions at different positions in these CDS clusters (NIF, PKNB, MRAZ, CHR and DNAX in Fig. 3). The essential role of the NIF locus in the interaction between *M. agalactiae* and host cells has been demonstrated previously by complementation studies [20].

The location of these CDS clusters can differ considerably from one mycoplasma species to another, even within the same phylogenetic group (Fig. S1, S2, and S3). In *M. agalactiae*, promoter sequences initiating the transcription of *nifS* were located outside of the NIF locus, with a sequence identified within the 3′ end of the *pip* gene (see above). Interestingly, the *pip* gene does not always co-localize with the NIF locus in other mycoplasma species, suggesting that this locus is inserted within transcriptionally active regions. Thus, it is tempting to speculate that some of the genes involved in the interaction between *M. agalactiae* and host cells may have been hitchhiking across evolution for transcriptionally active regions of the mycoplasma chromosome. Further evidence is needed to support this hypothesis.

In conclusion, cell culture provides a simple and efficient screening system for genome-scale analysis of mycoplasma loci

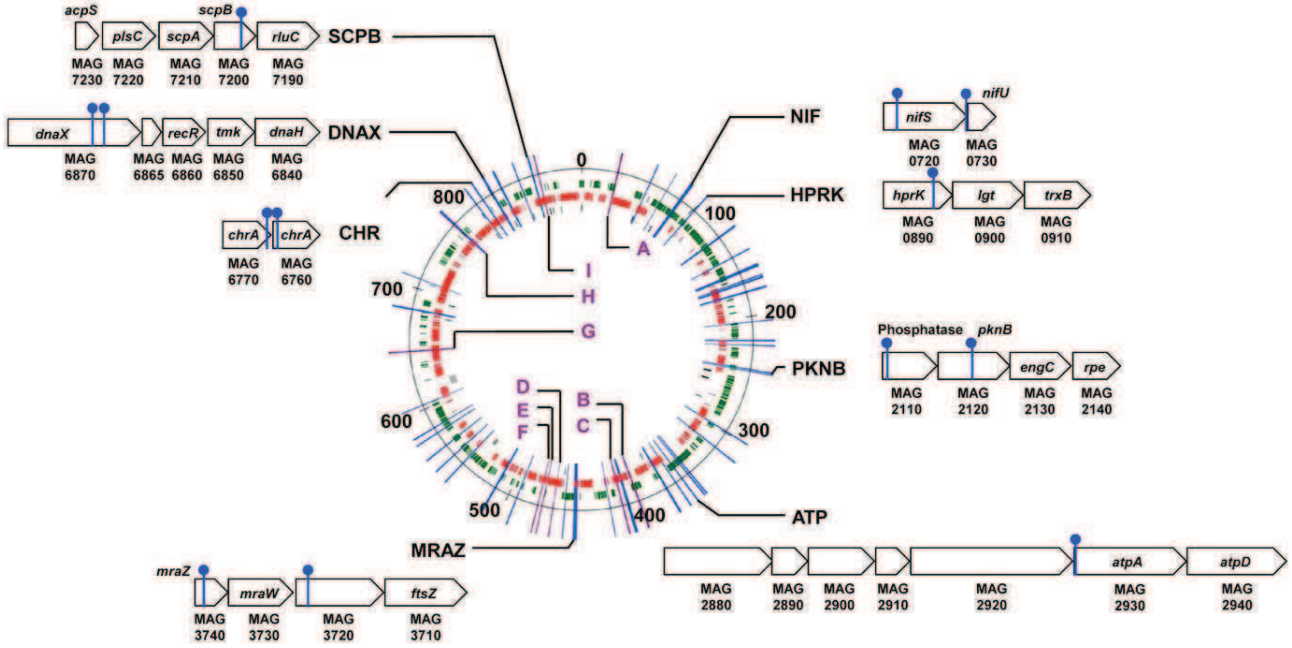

Figure 3. Genomic loci carrying transposon insertions in *M. agalactiae* mutants displaying reduced growth capacities in cell culture. Map of the 62 genomic regions found to be disrupted in *M. agalactiae* mutants selected on TIGMEC and/or TIGEF cells, produced using the Artemis genome browser and annotation tool [39]. Insertion sites found within CDS regions are indicated by a blue bar, while intergenic regions are designated by a letter code and a purple bar. Short CDS clusters are designated by capital letters and a schematic illustration of the locus, in which insertion sites are indicated by a filled circle on top of a blue bar. Genes are labeled. CDSs for hypothetical proteins of unknown function are labeled with their gene number. Genomic distances are indicated in kbp. *M. agalactiae* CDSs are colored in green (positive) or red (negative) on the chromosome to indicate their orientation. Non-coding RNAs are colored in black.
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contributing to host-pathogen interactions. This global approach, when combined with *in vivo* studies, can be an efficient strategy for identifying key factors involved in mycoplasma virulence and host-colonization, as well as a way to understand pathogenic processes involved in disease caused by these unconventional pathogens. The absence of small animal models for *M. agalactiae* and other ruminant mycoplasmal pathogens has been a significant bottle-neck for functional genomics studies using large mutant libraries. High-throughput screening of more than 2000 individual clones by co-culture with host cells revealed 62 loci in the genome of *M. agalactiae* that were required for growth in this environment. The relevance of these loci in the biology of *M. agalactiae* when it replicates in its natural host remains to be determined, but experimental infections in lactating ewes have confirmed the essential role played by *nifS* in host-colonization (data not shown). Since the specific cell line was shown to influence which mutants were selected, comparative studies with different cell types, including differentiated monolayer cultures and cellular players involved in the immune response to infection, may provide further information about the interactions of mycoplasmas in these different cellular environments. Finally, these results provide an experimental framework for the development of control strategies, based on attenuated live vaccines, against mycoplasmosis in ruminant species.

Supporting Information

Figure S1 Genomic position of short CDS clusters NIF, HPRK, MRAZ and CHR in mycoplasma species with sequenced genomes. Gene organization of the conserved CDS clusters identified during the screening was analyzed using the Microbial Genome Database for Comparative Analysis software [40]. Homologues genes are indicated by the same color code and a black bar. Three examples were chosen for each phylogenetic group. In the case of the CHR locus, no homolog was identified in the Pneumoniae group. MA: *M. agalactiae*, MMOB: *M. mobile*, MYPU: *M. pulmonis*, MLEA: *M. leachii*, MmmSC: *Mycoplasma mycoides* subsp. *mycoides* SC, MCAP: *M. capricolum* subsp. *capricolum*, UPAR: *U. parvum*, UURE: *U. urealyticum*, MGAL: *M. gallisepticum*.
(TIF)

Figure S2 Genomic position of short CDS clusters PKNB and ATP in mycoplasma species with sequenced genomes. Gene organization of the conserved CDS clusters identified during the screening was analyzed using the Microbial Genome Database for Comparative Analysis software [40]. Homologues genes are indicated by the same color code and a black bar. Three examples were chosen for each phylogenetic group. MA: *M. agalactiae*, MMOB: *M. mobile*, MYPU: *M. pulmonis*, MLEA: *M. leachii*, MmmSC: *Mycoplasma mycoides* subsp. *mycoides* SC, MCAP: *M. capricolum* subsp. *capricolum*, UPAR: *U. parvum*, UURE: *U. urealyticum*, MGAL: *M. gallisepticum*.
(TIF)

Figure S3 Genomic position of short CDS clusters DNAX and SCPB in mycoplasma species with sequenced genomes. Gene organization of the conserved CDS clusters identified during the screening was analyzed using the Microbial Genome Database for Comparative Analysis software [40]. Homologues genes are indicated by the same color code and a black bar. Three examples were chosen for each phylogenetic group. MA: *M. agalactiae*, MMOB: *M. mobile*, MYPU: *M. pulmonis*, MLEA: *M. leachii*, MmmSC: *Mycoplasma mycoides* subsp. *mycoides* SC, MCAP: *M. capricolum* subsp. *capricolum*, UPAR: *U. parvum*, UURE: *U. urealyticum*, MGAL: *M. gallisepticum*.
(TIF)

Table S1 Degree of homology of CDS disrupted in *M. agalactiae* mutants with other ruminant mycoplasma species. [a]CDS found disrupted in *M. agalactiae* growth-deficient mutants [6]. [b]Hypothetical proteins (HP) have no homolog outside the *M. agalactiae* species. Conserved hypothetical proteins (CHP) share sequence similarity with proteins of unknown function identified in mollicutes or other bacteria. COG categories of encoded proteins are indicated in parenthesis [38]. [c]Protein localization was predicted using TMHMM [40]; membrane (M), cytosolic (C), or indirectly linked to the membrane (IM). [d]Genes supposed to have undergone horizontal gene transfer (HGT) between M. agalactiae and mycoplasmas from the mycoides cluster [6] are indicated by a plus sign (+). Genes displaying homologies with sequences in *M. gallisepticum* or *M. synoviae* genomes and supposed to have undergone HGT between these two species are identified by a cross sign (x) [6]. [e]Percentages of identity and similarity were determine by local BLAST using Molligen [42]. [f]MCAP stands for *M. capricolum subsp. capricolum*.
(DOC)

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Author Contributions

Conceived and designed the experiments: AS CC EB. Performed the experiments: AS M-CH ES SG EB. Analyzed the data: AS CC EB. Contributed reagents/materials/analysis tools: SG. Wrote the paper: AS CC EB.

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AUTHOR: Agnès SKAPSKI

TITLE: *Mycoplasma agalactiae* functional genomic study: Analysis of virulence factors under cell culture conditions

ABSTRACT

Mycoplasma agalactiae is the etiological agent of contagious agalactia, a disease of small ruminants that is listed by the OIE. A cell culture assay was developed for the high-throughput analysis of the interactions between *M. agalactiae* and host cells. This approach identified 62 loci potentially involved in host-mycoplasma interactions, most of them encoding membrane proteins. Moreover, the NIF locus, a virulence factor involved in the synthesis of iron-sulfur cluster in several pathogenic bacteria, was found as essential for *M. agalactiae* proliferation in cell culture, while dispensable for axenic growth. Remarkably, this study also revealed that intergenic regions may play unexpected roles in mycoplasma-host interactions. These results provide a new approach to study pathogenic processes in mycoplasma infections and new means for the development of efficient vaccine strategies adapted to these unconventional pathogens.

KEY WORDS

Mollicutes, *Mycoplasma agalactiae*, contagious agalactia, ruminant, pathogen, cell culture, virulence, transposon mutagenesis, non-coding regions, iron-sulfur clusters, minimal genome.

AUTEUR : Agnès SKAPSKI

TITRE : Exploration fonctionnelle du génome de *Mycoplasma agalactiae* : Recherche des facteurs de virulence en culture cellulaire

DIRECTEURS DE THESE : Christine CITTI et Eric BARANOWSKI

LIEU ET DATE DE SOUTENANCE : Ecole Nationale Vétérinaire de Toulouse, le 10 novembre 2011

RESUME

Mycoplasma agalactiae est l'agent étiologique de l'agalactie contagieuse des petits ruminants, maladie inscrite sur la liste de l'OIE. Le développement d'un système de criblage en culture cellulaire a permis de mener une étude à haut-débit des interactions entre *M. agalactiae* et les cellules de l'hôte. Au cours de cette étude, 62 loci potentiellement impliqués dans les interactions hôte-mycoplasme ont été identifiés, une majorité codant pour des protéines membranaires. Par ailleurs, le locus NIF, un facteur de virulence impliqué dans la formation des groupes fer-soufre chez certaines bactéries pathogènes, s'est révélé essentiel pour la croissance de *M. agalactiae* en culture cellulaire. Cette étude montre également le rôle important que pourraient jouer certaines régions intergéniques dans les interactions hôte-mycoplasme. Ces résultats offrent de nouvelles pistes pour la compréhension des mécanismes de virulence chez les mycoplasmes et des outils pour le développement de stratégies vaccinales adaptées à ces pathogènes atypiques.

MOTS-CLE

Mollicutes, *Mycoplasma agalactiae*, agalactie contagieuse, ruminant, pathogène, culture cellulaire, virulence, mutagénèse transpositionelle, région non-codante, groupes fer-soufre, génome minimal.

DISCIPLINE ADMINISTRATIVE : Pathologie, Toxicologie, Génétique et Nutrition

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INTITULE ET ADRESSE DU LABORATOIRE

Equipe « Mycoplasmose des ruminants, pathologie mammaire et sécurité du lait »

Unité Mixte de Recherche 1225, « Interactions Hôtes Agents Pathogènes », INRA, ENVT

Ecole Nationale Vétérinaire de Toulouse

Pathologie de la Reproduction

23 chemin de Capelles, BP 87614

31076 Toulouse Cedex 3