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Ciclo XXXIII

Genome editing using the CRISPR/Cas9 system: an approach to improve the development of novel tools to control infectious diseases

Tesi presentata da: Dott.ssa Nicoletta Schibeci Natoli Scialli

Tutor: Prof. Sergio Rosati

Coordinatore del dottorato: Prof.ssa Maria Teresa Capucchio

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Abbreviations

AR	Antibiotic resistance
BoHV1gE	Glycoprotein E of Bovine Herpes Virus tipe 1
CA	Contagious agalactia
Cas	CRISPR-associated
Cas9	CRISPR associated protein 9
CH	Constant heavy chain
CL	Constant light chain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	pre-CRISPR RNA
DAB	3,3′-diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DSB	Double stranded break
dsDNA	Double-stranded DNA
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FluD	Influenza D virus
gE	Glycoprotein E
GFP	Green fluorescent protein
gRNA	Guide RNA
HDR	Homology directed repair
HR	Homologous recombination
HRP	Horseradish peroxidase
HGT	Horizontal gene transfer
IBR	Infectious bovine rhinotracheitis
ICES	Integrative conjugative elements
IGS	Immunoglobulins
IPTG	isopropyl b-thiogalactopyranoside
IS	Insertion sequence

LB	Luria–Bertani
mAb	Monoclonal antibody
MCS	Multiple cloning site
MC	*Mycoplasma canis*
MA	*Mycoplasma agalactiae*
NGS	Next generation sequencing
NHEJ	Non-homologous end-joining
PAM	Protospacer adjacent motif
PAC	Puromycin N-acetyltransferase
PEG	Polyethylene glycol
RNP	Ribonucleoprotein-complex
sgRNA	Single guide RNA
ssDNA	Single stranded DNA
TALEN	Transcription activator-like effector nuclease
*tet*M	Tetracycline resistance gene
tracrRNA	trans-activating crRNA
TREC	Tandem Repeats coupled with Endonuclease Cleavage
VH	Variable heavy chain
VL	Variable light chain
VLP	Viral-like particle
Vpma	Variable protein of the *M. agalactiae*
ZFN	Zinc-finger nuclease

Objectives of thesis

The control of infectious diseases is a fundamental aspect of both human and veterinary medicine. Many diseases have severe consequences on public health, as well as on the economy. Vaccination, in combination with rapid, precise and highly sensitive diagnostic tests are necessary for the control and treatment of disease. During the last decade, diagnostics for infectious diseases have vastly improved thanks to technological advancements. The detection of antigens, antibodies or nucleic acid molecules now plays a central role in the development of tools and specific tests for the routine surveillance of important human diseases, such as tuberculosis, malaria (Maltha et al., 2013; Weyer et al., 2013) and the recent worldwide COVID-19 pandemic (Graham, 2020; Zhang et al., 2020), as well as veterinary diseases, such as contagious agalactia (CA), infectious bovine rhinotracheitis (IBR), and so on (Rosati *et al.*, 1999, 2000; Bertolotti et al., 2015; Muratore et al., 2017; Colitti et al., 2018).

The development of new diagnostic tools for disease control purposes is generally a long and complex process. It requires the employment and development of numerous methods and protocols, such as gene editing, protein production, ELISA, the analysis of immunological parameters, both in vitro and in vivo testing, and so on. Furthermore, there is still a lot to explore in order to improve their capacity to preserve both human and animal health.

The principal aim of this research project was to explore the use of the CRISPR/Cas9 system for prokaryotic and eukaryotic genome editing with the end scope of developing novel diagnostic tools for veterinary applications. This system has great potential for development as it overcomes the issue of random integration, and it permits precise modification of the genome (Jinek et al., 2012; Cong et al., 2013; Black et al., 2017; Parthiban et al., 2019). In this study, we focus on the genome editing of two different subjects, a hybridoma cell line and *Mycoplasma agalactiae* (MA).

Hybridomas are immortalized cell lines widely used for the production of monoclonal antibodies (mAb) (Köhler & Milstein, 1975). They have an extensive endoplasmic reticulum that endows them with the capacity to secrete large quantities of more than one protein into the medium contemporaneously (the heavy and light chains of immunoglobulin). Thus, hybridomas provide an ideal mammalian expression system for the stable expression of one or two recombinant proteins at the same time.

We evaluated the application of the CRISPR system for the production of a stable eukaryotic system able to express and secrete high amounts of proteins of interest. We successfully edited the genome of a hybridoma cell line (3E1) using the CRISPR/Cas9 system. Specifically, first we replaced part of the immunoglobulin light chain with the Green Fluorescent Protein (GFP) gene to optimize the method for the purpose of this study and in order to obtain a precise knock-in. In a second phase, using the same approach, we included the gene for a protein of diagnostic interest in the donor DNA: the viral glycoprotein E of Bovine Herpes Virus tipe 1 (BoHV1) – the causative agent of respiratory disease and genital disorders in different animal species. Successful expression of the GFP and BoHV1gE/GFP proteins in the hybridoma clones 1D11 and 1E2, respectively, was achieved.

The production of a protein of diagnostic interest forms the starting point in the development of tools for disease control. Since recombinant glycoprotein E of Bovine Herpes Virus tipe 1 (BoHV1gE) expressed in mammalian system has been successfully used in assay format for IBR surveillance program in a region of north-west Italy (Piedmont) (Bertolotti et al., 2015; Muratore et al., 2017; Colitti et al., 2018), the development of a stable system for the high-yield expression of this protein, would represent a significant advance in the development of tools for IBR disease control.

These pilot experiments form the fundamental first step towards achieving the constitutive high-level expression of a protein of diagnostic interest – i.e. required to advance the proof of concept towards more specific clinical applications.

MA is the etiological agent of CA in sheep and goats. CA is controlled through either vaccination campaigns or the use of diagnostic tools based on recombinant proteins. A number of inactivated or live attenuated vaccines are available against CA, but no single vaccine is presently being universally adopted. Moreover, through the use of these kinds of vaccine, it is not possible to distinguish between the immunological responses of infected animals compared to those of vaccinated animals. A step forward in the control of this disease could be made through the development of marker vaccines. The basis of a marker vaccine is that vaccinated animals induce a different antigenic response compared with infected animals because a specific marker protein, detectable using specific diagnostic tools (e.g. ELISA), is not present in vaccine (Henderson, 2005). Therefore, the deletion or the replacement of a good 'marker' of infection constitutes the necessary starting point for the development of a marker vaccine.

Here, we explored the use of the CRISPR system to modify the MA genome with the goal of obtaining a prototype marker vaccine. Many commercially diagnostic tests employ the MA membrane lipoprotein p48, so the replacement of this protein was known to hold good potential for the development of a marker vaccine. Here, we focused our efforts on two markers of CA infection: the abovementioned p48 protein and the p40 protein. With regards to p48, first we attempted to replace the gene for this protein with an orthologous gene, and then, in a second attempt, with an

orthologous gene plus a gene for antibiotic resistance; for p40, we tried to substitute the gene with one for antibiotic resistance.

Obiettivi della tesi

Da sempre il controllo delle malattie infettive rappresenta un aspetto fondamentale della sanità pubblica umana e veterinaria. Infatti, molte patologie, se fuori controllo, oltre a causare serie conseguenze per la salute pubblica, rischiano di impattare seriamente anche sull'economia. Le vaccinazioni, in combinazione con test diagnostici rapidi, precisi e altamente sensibili, risultano essere fondamentali per il controllo ed il trattamento di queste patologie a carattere infettivo. Grazie alle scoperte scientifiche e agli avanzamenti tecnologici dell'ultimo decennio, la diagnostica applicata alle patologie infettive ha subito una grande crescita. Indubbiamente, il rilevamento e la produzione di antigeni, anticorpi o acidi nucleici, ha avuto un ruolo centrale nello sviluppo di test specifici per la sorveglianza di routine di importanti patologie umane, come ad esempio la tubercolosi, la malaria (Maltha et al., 2013; Weyer et al., 2013), oppure la recente pandemia da COVID-19 (Graham, 2020; Zhang et al., 2020), ma anche per il controllo di patologie veterinarie, come ad esempio l'agalassia contagiosa, la rinotracheite infettiva del bovino ed altre ancora (Rosati et al., 1999, 2000; Bertolotti et al., 2015; Muratore et al., 2017; Colitti et al., 2018).

Lo sviluppo di nuovi test diagnostici, per il controllo delle patologie, è un processo molto lungo e complesso che richiede l'utilizzo, ma anche lo sviluppo e l'ottimizzazione, di molti metodi e test, come ad esempio l'editing genetico, la produzione di proteine, l'analisi di parametri immunologici, ELISA, test in vivo ed in vitro, e così via. Infatti, nonostante gli innumerevoli avanzamenti della ricerca scientifica, c'è ancora molto da scoprire e migliorare per il benessere della salute sia umana che animale.

In questo progetto di ricerca, il sistema CRISPR/Cas9 è stato impiegato per l'editing genomico sia di procarioti che di eucarioti al fine di sviluppare nuovi test diagnostici da utilizzare in ambito veterinario. Il sistema CRISPR/Cas9 è una tecnologia all'avanguardia, con un grandissimo potenziale nell'ambito dell'ingegneria genetica ed uno dei principali vantaggi di questo sistema è quello di modificare in maniera molto precisa il genoma, evitando modifiche random (Jinek et al., 2012; Cong et al., 2013; Black et al., 2017; Parthiban et al., 2019). In particolare, ci siamo focalizzati sull'editing genomico di due soggetti: una linea cellulare di ibridomi e *Mycoplasma agalactiae* (MA).

Gli ibridomi sono una linea cellulare immortalizzata, ampiamente utilizzati per la produzione di anticorpi monoclonali (Köhler & Milstein, 1975). Gli libridomi hanno un reticolo endoplasmatico molto ben sviluppato, che permette loro di secernere nel terreno di coltura grandi quantità di una o di più

proteine allo stesso tempo (le catene leggere e pesanti delle immunoglobuline). Per questo motivo, gli ibridomi possono essere considerati una linea cellulare eucariota ideale per l'espressione contemporanea di una o più proteine ricombinanti.

In questo progetto di ricerca, la tecnologia di editing genomico CRISPR è stata utilizzata per cercare di ottenere un sistema eucariota capace di esprimere stabilmente e in grande quantità una proteina di interesse. In particolare, una specifica linea cellulare di ibridomi, chiamata 3E1, è stata modificata geneticamente con successo. In una prima fase del progetto, per ottimizzare il metodo, una parte della catena leggera dell'immunoglobulina è stata sostituita con il gene della Green Fluorescent Protein (GFP). In una seconda fase, al frammento di DNA, contenente il gene della GFP, è stato aggiunto il gene di una proteina d'interesse nel campo della diagnostica veterinaria: la glicoproteina E dell'Herpes Virus Bovino di tipo 1 (BoHV1gE), un virus responsabile di patologie respiratorie e genitali, soprattutto nei bovini, ma anche in altre specie di animali. In seguito all'editing genetico della linea 3E1 sono stati ottenuti due cloni, chiamati 1D11 e 1E2, capaci di esprimere la proteina GFP e la poliproteina BoHV1gE/GFP, rispettivamente.

Sicuramente, la produzione di una proteina d'interesse diagnostico rappresenta il punto di partenza per lo sviluppo di test da utilizzare nelle strategie di controllo delle malattie infettive. Attualmente, la BoHV1gE è utilizzata nella maggior parte dei test per il controllo e la sorveglianza della rinotracheite infettiva del bovino (Bertolotti et al., 2015; Muratore et al., 2017; Colitti et al., 2018). Per questo motivo, lo sviluppo di un sistema stabile, capace di esprimere grandi quantità di questa proteina, rappresenterebbe un importante passo avanti nello sviluppo di test per il controllo di questa patologia.

Questo studio pone le basi per lo sviluppo di un sistema eucariota stabile per l'espressione di grandi quantità di proteine d'interesse diagnostico, dalla sua prova di concetto fino a specifiche applicazioni cliniche.

MA è l'agente eziologico responsabile della agalassia contagiosa (AC) in pecore e capre. Attualmente l'AC è tenuta sotto controllo attraverso campagne di vaccinazione e grazie all'utilizzo di specifici test diagnostici. Contro l'AC sono disponibili sia vaccini inattivati, che vaccini vivi attenuati, tuttavia attualmente non è ancora disponibile un vaccino che sia universalmente utilizzato contro questa patologia. Inoltre, l'utilizzo dei sopraccitati vaccini ha un limite importante, che incide negativamente sull'eradicazione di questa patologia; questi vaccini non consentono di distinguere tra animali infetti e vaccinati. Date queste premesse, è chiaro che lo sviluppo di un vaccino marker costituirebbe un grande progresso nel controllo di questa importante patologia. Infatti, il principio dei vaccini marker è quello di indurre negli animali vaccinati una risposta antigenica differente, rispetto a quella degli animali infetti, perché una specifica proteina (definita appunto marker di infezione), individuabile attraverso l'utilizzo di test diagnostici (ad esempio ELISA), non è presente nel vaccino

marker (Henderson, 2005). Quindi, l'eliminazione, o la sostituzione, di un buon marker di infezione è una fase fondamentale nel lungo processo di sviluppo di un vaccino marker.

L'obiettivo di questo progetto di ricerca è stato quello di testare il sistema CRISPR per l'editing genomico di MA, con lo scopo finale di ottenere il prototipo di un vaccino marker. Molti test diagnostici, attualmente in commercio per la diagnosi di AC, si basano sull'utilizzo della p48, una lipoproteina di membrana di MA. Per questo motivo, l'eliminazione o la sostituzione del gene che codifica questa proteina potrebbe rappresentare un gran potenziale nello sviluppo di vaccini marker. In particolare, questa ricerca si è focalizzata sulla p48 e sulla p40, un'altra proteina di membrana di MA, anch'essa considerata un ottimo marker d'infezione dell'AC. Per quanto riguarda la proteina p48, in una prima fase del progetto l'obiettivo è stato quello di sostituire il gene di questa proteina con il gene di una proteina ortologa. In un secondo momento, al gene della proteina ortologa è stata aggiunta un'antibiotico-resistenza. Invece, per quanto riguarda la p40, l'obiettivo principale è stato quello di sostituire il gene di questa proteina con un'antibiotico-resistenza.

Contents

Chapter 1
The CRISPR/Cas system

1.1 Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) are a family of DNA sequences found in the genomes of prokaryotic organisms that play an integral role in the adaptive immune response of these organisms against bacteriophages, viruses and other foreign genetic elements (Hille et al., 2018). The CRISPR loci are composed of a CRISPR array plus a set of CRISPR-associated (Cas) genes that precedes it. CRISPR arrays comprise two principal elements: highly conserved repeated sequences, known as "repeats", and non-repetitive sequences, called "spacers" (Ishino et al., 1987; Jansen et al., 2002; Barrangou et al., 2007). Over recent decades, the CRISPR system has been exploited in numerous ways within the realm of genome editing. The CRISPR/Cas9 system is a powerful genome editing tool, with many applications in different fields of biology (Mei et al., 2016; Zhang et al., 2018; Pickar-Oliver & Gersbach 2019; Wu et al., 2019). The principal advantages of the CRISPR/Cas9 system regard its high editing precision, its low cost, and the fact that it is simple to use and generates results quickly. Furthermore, its high efficiency and the possibility of obtaining simultaneous multiple DSB with two or more sgRNA are additional advantages of the CRISPR/Cas9 system compared with other tools for genome editing, such as transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) (Gaj et al., 2013; Ran et al., 2013).

1.1.1 The discovery of CRISPR

It was in the 1980s when various research groups first identified CRISPR (Stern et al., 1984; Ishino et al., 1987; Newburg et al., 1987; Nakata et al., 1989). However, at that time, the biological significance of these repetitive sequences, then called REP, was not known due to the lack of suitable technologies for genome sequencing. In the same decade, research revealed REPs to be present in many different species of microorganisms, and they were initially used for the genetic typing of bacteria (Nakata et al., 1989; Hermans et al., 1991; Groenen et al., 1993; Mojica et al., 1993 and 1995; She et al., 2001; Zivanovic et al., 2002). However, in 2000 Mojica and colleagues finally realized that the presence of these repetitive sequences, present in many distantly related microorganisms, held important biological significance (Mojica et al., 2000).

In 2002, for the first time, Jansen and colleagues called this family of repetitive DNA sequences "clustered regularly interspaced short palindromic repeats" (Jansen et al., 2002). Since then, the name CRISPR has become unanimously accepted by the whole scientific community, thus avoiding any possible confusion through the use of multiple terms.

In 2005, three different research groups contemporaneously discovered that the origin of spacers could be traced to bacteriophages, viruses and plasmids. For this reason, they proposed the hypothesis that CRISPR has a role in adaptive immunity in bacteria (Mojica et al., 2005; Pourcel et al., 2005; Bolotin et al., 2005). However, it was only two years later that Barrangou and colleagues provided the evidence that confirmed this hypothesis (Barrangou et al., 2007). In this research, they showed that modification of the CRISPR locus, e.g. by removing or adding particular spacers, modified the resistance of the bacterium towards phage infections. Consequently, it became clear that phage resistance was correlated with the modification of spacers, and therefore that CRISPR constitute the backbone of the adaptive immune system in prokaryotes.

The CRISPR system of *S. thermophilus* was the first to be expressed in *E. coli* (Sapranauskas et al., 2011). This system conferred resistance to *E. coli* against plasmid transformation and phage infections. This research was critical because it proved, for the first time, that the CRISPR system can be transferred across distant genera. This discovery was the starting point for the use of this system as a genome editing tool (Jinek et al., 2012).

1.1.2 CRISPR system classification

Different CRISPR systems exist and are divided in two major classes: class 1 and class 2. These two classes differ in effector molecule characteristics: in class 1 the effector molecule contains multiple subunits, whereas in class 2 there is a single subunit effector (Makarova et al., 2015). The CRISPR systems are further divided into six different types, annotated by roman numerals: I-VI. Type I, III and IV belong to class 1, whereas type II and V belong to class 2 (Chylinski et al., 2014). Type VI has yet to be characterized and thus classified (Wright et al., 2016).

1.1.3 CRISPR system immunity mechanism

The mechanisms underlying CRISPR immunity can be divided in three different steps: adaptation, expression and interference (Figure 1).

In the adaptation stage, short DNA sequences from viruses, phages or plasmids are inserted into the CRISPR array as spacers. The key proteins involved in this stage are thought to be Cas1 and Cas2 – the two Cas proteins that are present in all CRISPR/Cas systems (Makarova et al., 2011).

During the expression stage, the CRISPR array is expressed with the consequent production of pre-CRISPR RNA (crRNA). Pre-crRNA modification varies depending on the CRISPR type, but for all types the final product is a short crRNA that contains the target sequence complementary to the foreign DNA sequence.

The interference stage comes into play in the case of a second invasion by the same agent. When this occurs, the crRNAs produced as a result of the initial exposure to the infectious agent guide Cas nucleases to the foreign DNA. If the invading nucleic acid contains a target sequence complementary to the crRNA target, and if it is flanked by a short sequence called a protospacer adjacent motif (PAM), then the Cas protein cleaves the foreign DNA (Jinek et al., 2012, 2014). The presence of the PAM sequence adjacent to the target sequence in the genome was found to be necessary in order to obtain a double strand break (DSB) (Jinek et al., 2012). Notably, the PAM sequence is present in the invading DNA, but it is not present in the CRISPR locus.

In 2012, two different teams isolated the complex formed by the fusion of the CRISPR-associated (Cas) protein and a guide RNA (gRNA) (Gasiunas et al., 2012; Jinek et al., 2012). These studies were the first to demonstrate that this complex, a ribonucleoprotein (RNP), cleaves the invading DNA by means of a DSB near to the target sequence.

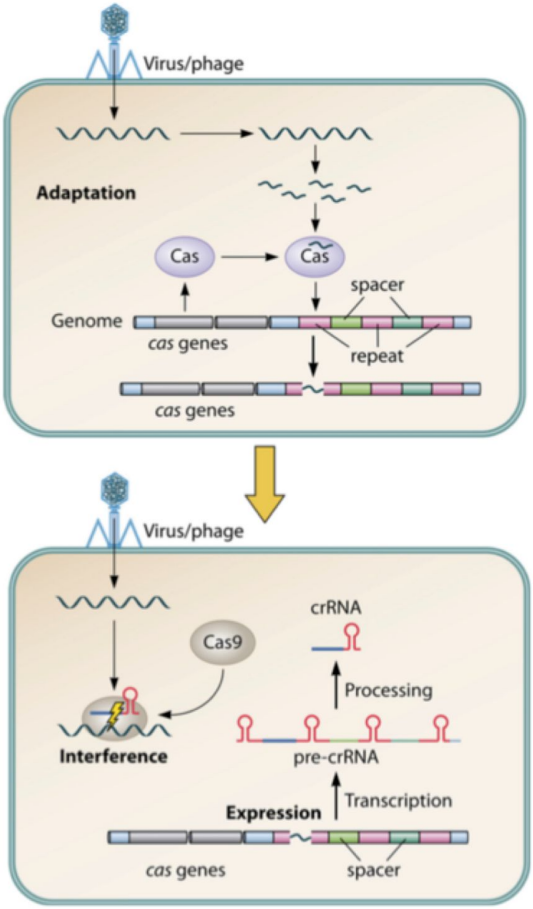

Figure 1: (See legend on next page)

Figure 1: Picture from Ishino et al., 2018. Schematic representation of the CRISPR system immunity mechanism. Short DNA sequences from the invading virus, phage or plasmids are incorporated into the CRISPR array. In the case of a second invasion by the same agent, pre-crRNA is expressed and processed to obtain the crRNA. Finally, the crRNA sequences function as guides for the Cas nuclease (Cas9 in the CRISPR type II system) that cleaves the invading DNA.

1.1.4 The type II CRISPR system

The type II CRISPR system (also called CRISPR/Cas9) is the most well characterized (Jinek et al., 2012). In this system, the endonuclease protein is Cas9, and the gRNA is formed by the fusion of the crRNA and the trans-activating crRNA (tracrRNA). The tracrRNA is a small noncoding RNA; it does not contain a target sequence, but it is fundamental for the binding of the gRNA to the Cas9 protein, whereas the crRNA (that contains the target sequence) alone is unable to guide Cas protein to the invading DNA in order to cleave it.

Under natural circumstances, the crRNA and the tracrRNA are initially separate, they then come together to make a base-paired structure (tracrRNA:crRNA) that forms a complex with the Cas9 protein (Figure 2-a). The type II CRISPR system is the most well characterized and most widely exploited tool in both eukaryotic and prokaryotic genome editing (Jinek et al., 2012; Cong et al., 2013; Black et al., 2017; Parthiban et al., 2019; Selle & Barrangou, 2015; Li et al., 2015; Zhang et al., 2018).

1.1.5 The CRISPR/Cas9 system as tool for genome editing

The key components of the engineered CRISPR/Cas9 system include the Cas9 endonuclease and single guide RNA (sgRNA), which together form the RNP complex (Figure 2-b). The sgRNA is formed by the fusion of the crRNA and the tracrRNA. It is easy to design and fast to obtain. The Cas9 protein and the sgRNA can be delivered into target cells using different methods, such as vector-based expression systems or RNA transfection, or directly as a preformed Cas9/sgRNA complex. Compared with other methods, the use of RNP complexes reduces the off-target effect, and has been optimized for various cell lines using electroporation or lipid-mediated transfection techniques (Sander & Joung, 2014; Liang et al., 2015). Once delivered into a target cell, the RNP complex produces a targeted-DSB in a specific position of the genome, close to the PAM sequence.

Following the DSB, DNA repair takes place by means of one of two principal mechanisms: non-homologous end joining (NHEJ), or homologous recombination (HR) (Pannunzio et al., 2018; Wright et al., 2018). Following NHEJ, the result is the insertion or the deletion of bases, which produces micro insertion-deletion mutations (indels) or gene knockouts. Alternatively, DNA repair via HR involves a donor DNA sequence that contains two flanking regions (Wright et al., 2018). The combination of the CRISPR/Cas9 system with HR permits the insertion of a coding sequence of interest into a precise area of the genome; for example, in a region with high transcriptional activity to obtain high expression levels. In this case, a gene knock-in is obtained. The donor DNA can be in the form of a plasmid, double-stranded PCR-derived DNA (dsDNA) or a single-stranded DNA (ssDNA); use of the latter as donor increases the integration efficiency by HR (Renaud et al., 2016; Li et al., 2017).

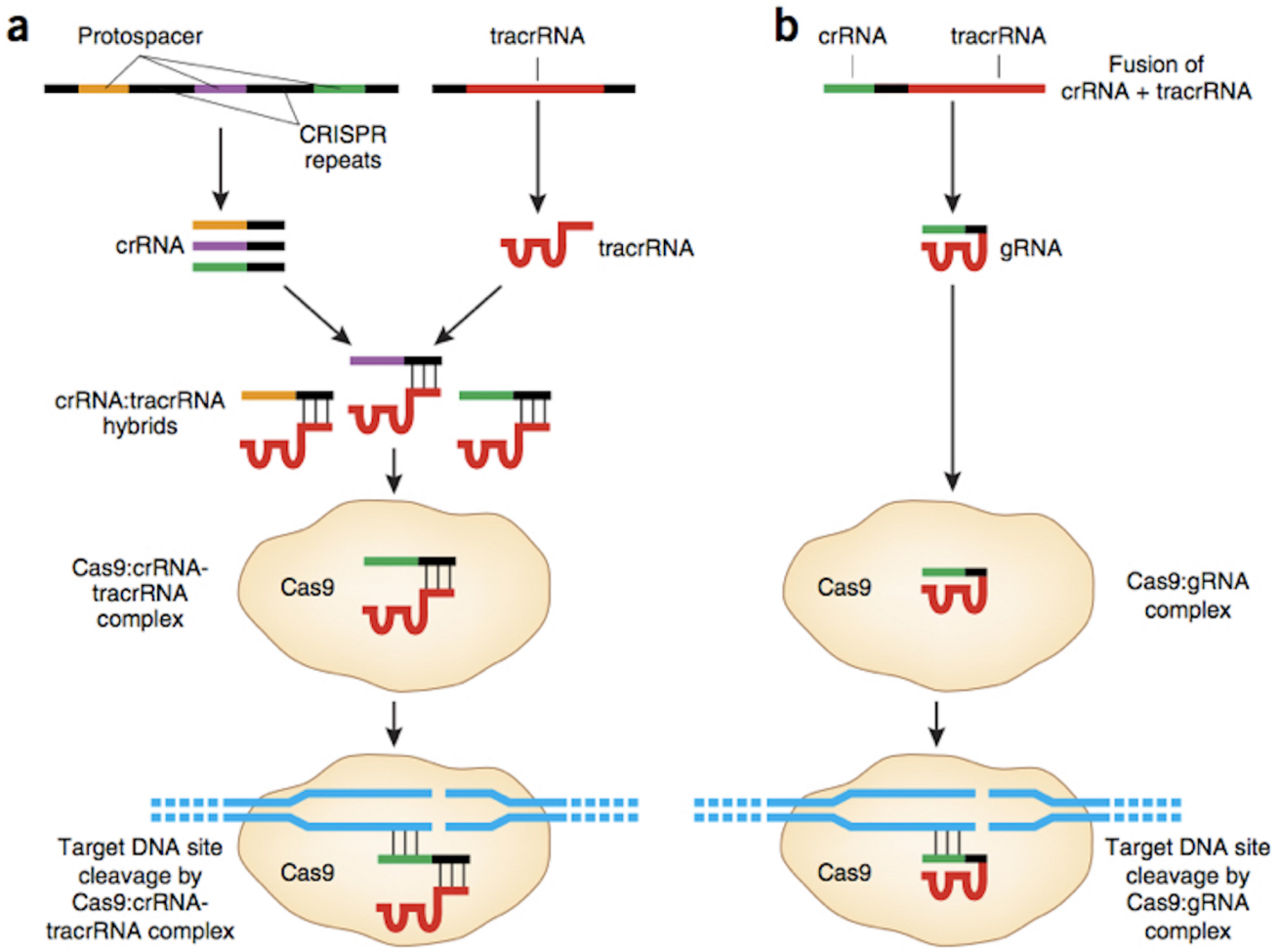

Figure 2: (See legend on next page)

Figure 2: Figure from Sander & Joung, 2014. CRISPR mechanism. (a) Short DNA sequences from viruses, phages or plasmids are inserted into the CRISPR array as "protospacers" (or spacers). Subsequently, the CRISPR array is expressed with the production of pre-CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The crRNA and the tracrRNA come together to make a base-paired structure (tracrRNA:crRNA) that forms a complex with the Cas9 protein. The crRNA-tracrRNA:Cas9 complexes recognize the foreign DNA and make a DSB bearing the protospacer sequences. (b) In the CRISPR-Cas9 system, crRNA and tracrRNA fuse together to form the guide-RNA (gRNA), which then forms a complex with the Cas9 nuclease and cleaves the DNA at the target site complementary to the 5′ 20 nt of the gRNA.

Chapter 2

Genome editing of a hybridoma cell line via the CRISPR/Cas9 system: a new approach for constitutive high-level expression of heterologous proteins in eukaryotic system

Abstract

The power of the CRISPR/Cas9 system has revolutionized genome editing in many fields of biology. These applications have expanded exponentially over recent years, including those regarding protein expression technologies. The CRISPR/Cas9 system avoids random integration of the gene of interest and due to this characteristic can be exploited to obtain a stable cell line for the high-yield expression of recombinant proteins. Here we propose a method to edit a hybridoma cell line for the constitutive expression of proteins of interest using the CRISPR/Cas9 system. First, with the scope of optimizing the method, we replaced part of the light chain of immunoglobulin with the Green Fluorescent Protein (GFP) gene, obtaining a precise knock-in in the hybridoma genome. We confirmed the expression and secretion of GFP into the culture medium via fluorimetric analysis, as well as correct genome editing by RNA sequencing. Then, using the same approach, we included the gene encoding a protein of diagnostic interest, the Bovine Herpesvirus 1 glycoprotein E, in the donor DNA. We obtained a stable clone able to secrete glycoprotein E protein in fusion with GFP into the culture medium. This result was confirmed by ELISA and Western Blot analysis. This study confirms the suitability of this cell line for the production of proteins of diagnostic interest by stable gene expression in a mammalian system. These experiments will enable the technique to be developed from its proof of concept to more specific applications in the field of infectious disease diagnostics.

2.1 Introduction

2.1.1 Hybridoma cell lines

Since their development in the 1970s, hybridomas have played an important role in monoclonal antibody (mAb) production (Köhler & Milstein, 1975). The first hybridoma cell line was generated by the fusion of B-lymphocytes with plasmacytoma (myeloma) cells, and it has the ability to express a considerable amount of a few proteins (e.g. the heavy and light chains of immunoglobulin) under the control of a strong promoter. Moreover, hybridomas are immortalized cell lines, making them easy to propagate in suspension culture (Köhler & Milstein, 1975; Pirofski et al., 1990). For these and other features, they be considered an ideal eukaryotic cell system for protein expression in which to attempt the CRISPR/Cas9 strategy for protein expression.

2.1.2 The genetic structure of immunoglobulins

Immunoglobulins (Igs) are Y-shaped glycoproteins. They are composed of two copies of identical heavy chains (~50 kDa each) and two copies of identical light chains (~25 kDa), joined together by disulphide bonds and non-covalent interactions. Both chains are divided into: a variable region (VH and VL for the heavy and light chains, respectively) that recognizes the target of the Ig; and a constant region (CH and CL), the conserved structure that specifies the isotype of the Ig.

Under natural circumstances, the diversity of the antibody repertoire is due to rearrangement events within the immunoglobulin genes via V(D)J recombination (Figure 3). The heavy chain genes are divided into variable (V), diversity (D), and joining (J) genes, upstream of the genes that make up the constant (C) region, whereas the light chain genes do not include any D region loci. Each chain is encoded by a specific gene complex, obtained after a series of gene rearrangement events. Following rearrangement, the light chain gene complex contains one V region out of 200, one J region out of 5 and only one C region. This rearrangement allele is transcribed, followed by RNA splicing to remove the introns and obtain a continuous exon (VJC). The final mRNA also contains a secretory signal peptide that allows for the membrane translocation of the Ig (Dorshkind & Rawlings, 2018).

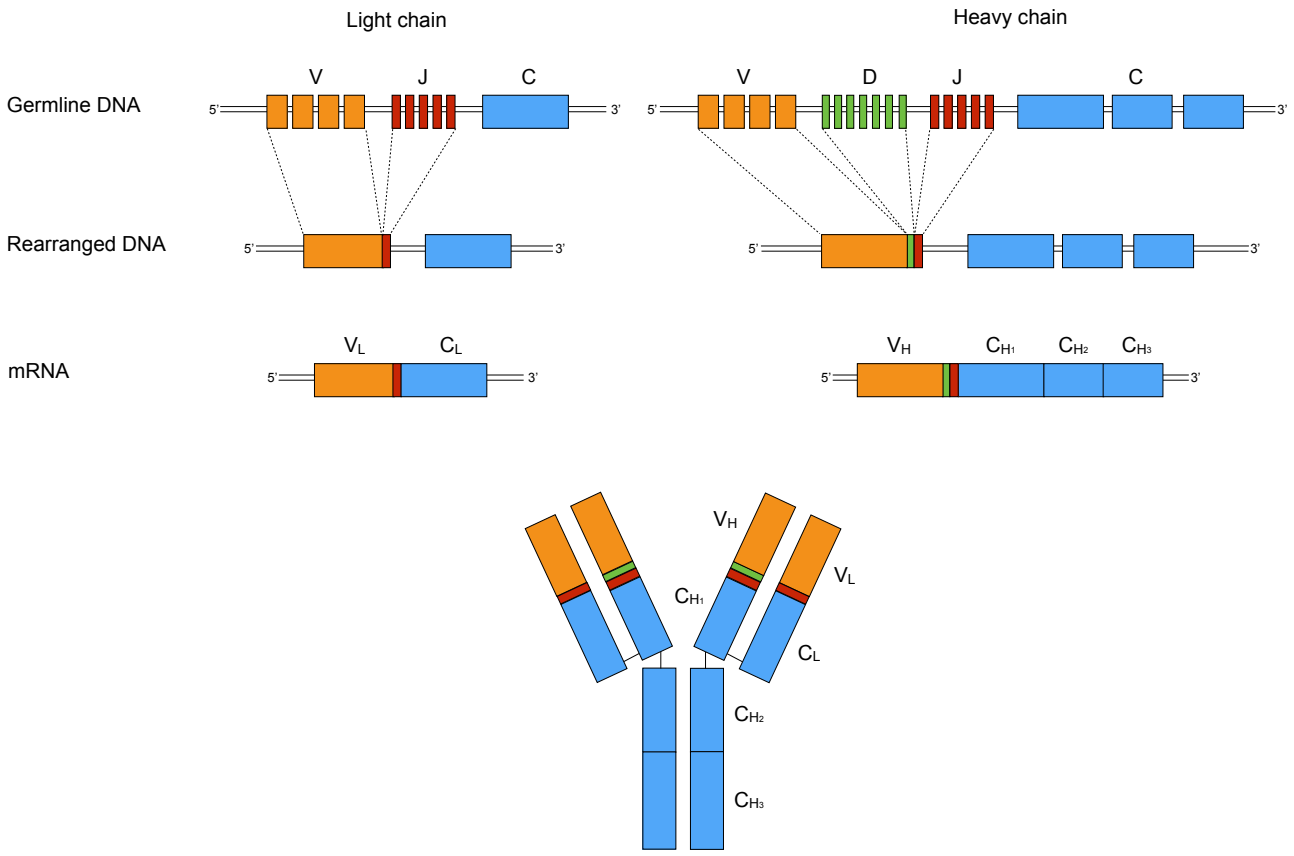

Figure 3: Schematic representations of the V(D)J recombination and immunoglobulin genetic structure. Different germline gene segments coding for the variable Ig heavy and light chains are joined by V(D)J recombination. The rearrangement allele is transcribed, followed by RNA splicing to remove the introns and obtain a continuous exon (VJC). Finally, the heavy and light chains fold into the typical Y-shaped immunoglobulin molecule and are linked by disulphide bridges.

2.1.3 Protein expression

Since its initial development in the 1970s, recombinant DNA technologies for heterologous protein expression have undergone enormous expansion. A great number of genes have been cloned and expressed into different systems to obtain recombinant protein to use for the development of diagnostic kits, therapeutics, vaccines and so on, for both human and animals. Indeed, recombinant protein expression is of paramount importance in a variety of fields, including, but not limited to, basic research, diagnostics and the pharmaceutical industry (Walsh, 2014, 2018; Khan et al., 2016). For example, in the context of the recent COVID-19 pandemic, recombinant DNA technology is

playing an indispensable role worldwide in the development of diagnostic kits, vaccines and pharmaceuticals against this infectious disease (Graham, 2020; Zhang et al., 2020).

Many different expression systems exist, involving prokaryotic, yeast, insect and mammalian cells, each with their specific advantages and disadvantages (Table 1). However, due to the importance of this field, researchers are continually trying to improve and optimize these systems further while simultaneously attempting to develop new strategies for the production of recombinant proteins

Table 1: Main advantages and disadvantages of the most used protein expression systems.

Protein expression system	**Advantages**	**Disadvantages**
Prokaryotic	Low cost; rapid expression time; high yield expression; easy scale-up.	Lack of post-translational modifications; possible presence of contaminants.
Yeast	Low cost; rapid expression time; high yield expression; various post-translational modifications possible.	Incorrect glycosylation; difficulty to express some secretory proteins.
Insect cell	Rapid expression time; various post-translational modifications possible; ability to express soluble proteins.	High cost; possible presence of contaminants; simpler N-glycosylation compared with mammalian cells.
Mammalian cell	Correct and complete post-translational modifications; correct protein assembly and folding; ability to express soluble proteins; transient and stable expression possible.	High cost; low yield; long expression time; hard to scale up.

2.1.4 Prokaryotic expression systems

Prokaryotic expression vectors are the most commonly used systems for the expression of heterologous proteins. They are preferred for a number of different reasons; they are more economical and simpler to use with respect to other systems, results are obtained quickly and they have the potential to produce high protein yields (Demain & Vaishnav, 2009; Zerbs et al., 2009). In particular, the *Escherichia coli* expression system is widely adopted, due to this bacterium's rapid growth, the detailed knowledge of its genome, and the wide variety of genetic tools available for fast

and easy protein expression and purification in this system (Rosano & Ceccarelli, 2014). The major limit of prokaryotic systems, however, is the lack of any post-translational modification of the expressed protein. Since post-translational modification is often essential for the correct folding of a protein, an eukaryotic expression system needs to be used when such modifications are required; for example, glycosylation improves protein stability and preserves the biological activity of the protein (Lalonde & Durocher, 2017).

2.1.5 Eukaryotic expression systems

The choice of eukaryotic host principally depends on the characteristics of the protein being expressed, since different hosts can permit different post-translational modifications to occur (Betenbaugh et al., 2004).

Yeast fall into the category of eukaryotic expression systems, and they have the characteristic of being able to combine the advantages of both prokaryotic and eukaryotic systems; namely, they can generate high expression levels of recombinant proteins, they are characterized by fast growth, the cost of growth media is low, and they are able to carry out most of the post-translational modifications on expressed proteins. However, yeast systems are not able to glycosylate expressed proteins correctly, and this continues to be a significant problem in the use of these systems. (Pasikowska et al., 2012).

Insect cells constitute another important system that conserves all the advantages of all the eukaryotic systems. However, this system, once again, albeit to a lesser extent than yeasts, produces differences in the glycosylation patterns with respect to those of mammalian cells.

Mammalian cells are, undoubtedly, the most preferred system of choice for protein expression, not only to ensure proper protein folding and all correct post-translational modifications, including glycosylation, but also because the system is capable of secreting the expressed protein into the medium and it is able to produce soluble proteins that are simpler to purify (Andréll & Tate, 2013). At present, the limited production yields and the difficulties experienced in genetic manipulation are the most significant drawbacks of the mammalian system for recombinant protein expression.

The mammalian protein expression system is usually divided into two categories: transient and stable expression. A mammalian transient protein expression system is simple, rapid and produces an acceptable yield of recombinant protein, but only for a short time (2-3 days). It is also expensive and expression is difficult to standardize. Consequently, the transient expression system is

best used for small-scale production and, due to the many variable factors that can influence the yields and protein quality, this method is not recommended when highly reproducible results are required (Wurm, 2004; Bollin et al., 2011). A stable protein expression system requires the integration of the gene of interest and the selection of a cell line capable of expressing the protein over time. However, the genetic modification of mammalian cells in most previously adopted techniques, required to obtain a stable protein expression system, generally involves the random integration of the gene of interest, sometimes resulting in its insertion into loci with variable levels of transcriptional activity (Büssow, 2015), resulting in very low product yields. Furthermore, the high cost and the long and laborious process for the generation of stable cell lines often make this system unfeasible in economic terms for academic applications or for small companies. The development of a strategy that permits the avoidance of random integration would constitute an important step forward in the development of a stable protein expression system.

2.1.6 The CRISPR/Cas9 system as a genetic tool in hybridoma cell lines

Recent studies successfully demonstrated the use of the CRISPR/Cas9 system combined with Homology Directed Repair (HDR) to edit the genome of hybridomas (Pogson et al., 2016; Parola et al., 2018; Parola et al., 2019). The aim of such studies is usually the modification of mAbs; for example, to obtain antibodies in a specific format, with the possibility of inserting tags or mutations, or to knock out the constant region in order to express an antibody fragment instead of the whole immunoglobulin (Cheong et al., 2016; Khoshnejad et al., 2018; van der Schoot et al., 2019).

Parola and colleagues, by using the CRISPR/Cas9 system, edited the genome of a hybridoma to generate stable antibody-producing cell lines. They used HDR to replace the variable heavy chain (VH) with a fluorescent reporter (mRuby), and, at the same time, they deleted the VL by NHEJ. As a result, this cell line could be modified by replacing the mRuby gene with a synthetic antibody scaffold, with the final aim of expressing and secreting a full-length specific antibody. Furthermore, they replaced the murine heavy constant region with the human one to express human antibodies. This research confirms the possibility of exploiting hybridoma cell lines for the production and secretion of modified antibodies by genome editing, using the CRISPR/Cas9 system.

In this context , it is clear how the specificity of the CRISPR/Cas9 system allows for the insertion of a coding sequence of interest into a precise region of the hybridoma genome, thereby avoiding random integration (Smith et al., 2014). Targeted insertion of a coding sequence into the

genome can considerably increase the protein expression level compared with random integration (Lee et al., 2015).

In this research, we focused on the immunoglobulin kappa light chain constant region. Our aim was to replace it with a gene of interest and exploit the original secretory pathway in order to express secreted recombinant protein into the medium. As a first step, we inserted the gene coding for GFP into the hybridoma genome, replacing the light chain constant region, to optimize the system for the purpose of this study and as a means to verify correct genome editing. In a second phase, we included our gene of interest – the viral glycoprotein E of Bovine Herpes Virus type 1 (BoHV1ge) – in the construct in order to demonstrate the applicability of the system in the field of infectious disease diagnostics.

2.1.7 Bovine herpesvirus 1

Bovine herpesvirus 1 (BoHV1) is an enveloped DNA virus belonging to the family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus (Roizman et al., 1992). This virus is the causative agent of respiratory disease (Infectious Bovine Rhinotracheitis - IBR), genital disorders (vulvovaginitis or balanoposthitis), transient infertility and abortion in cattle (Kahrs, 2001). Following the primary infection of the mucous membranes of either the respiratory or genital tract, the infection becomes latent, but may flare-up later on, especially in the case of stress or treatment with corticosteroids (Ackermann & Engels, 2006; Winkler et al., 2000). Although BoHV1-associated diseases are associated with low mortality rates, they can cause serious problems to animal health and livestock productivity (Raaperi et al., 2014).

A number of BoHV-1 vaccines are available to prevent and reduce the problems and economical losses associated with BoHV-1 infection (van Oirschot et al., 1999-b). In recent years, the majority of vaccines used to prevent and reduce the problems associated with BoHV1 infection are based on DIVA properties. Glycoprotein E (gE) deleted marker vaccines are commonly used in combination with specific gE ELISA protocols to distinguish between infected and vaccinated animals (Mars et al., 2001; Rijsewijk et al., 1999).

The most common approach to recombinant gE production involves the transient plasmid transfection of mammalian cells (Bertolotti et al., 2013). This method is rapid, but generally used for small-scale productions only since protein expression is limited to just a few days, and it is not recommended when highly reproducible results are required (Wurm, 2004; Bollin et al., 2011). Recombinant gE expressed in mammalian system has been successfully used in assays, for the detection of IgG against the BoHV1gE on pooled milk samples, deployed as part of IBR surveillance

program in a region of north-west Italy (Piedmont) (Bertolotti et al., 2015; Muratore et al., 2017; Colitti et al., 2018). However, the high-yield expression of recombinant gE in a stable cell system, like that provided by hybridomas, would constitute a significant step forward in the development of new tools for the control of IBR. Hence, the aim of this study is to develop such a strategy from its proof of concept to more specific diagnostic applications.

2.2 Materials and methods

2.2.1 Hybridomas selection and culture conditions

The hybridoma cell line 3E1 used in this work, developed in a previous study, is known to secrete a monoclonal antibody specific to the hemagglutinin-esterase-fusion protein of influenza D virus (mAb-FluD) (Moreno et al., 2019). The 3E1 cell line derived from the fusion partner NS0 - the murine myeloma cell line originally used for hybridoma production. The NS0 was included in the genetic analysis as reference. Hybridoma cells were cultured in a CO_2 incubator (5% CO_2) at 37°C, in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 2% L-glutamine (Sigma-Aldrich), 1% Antibiotic-Antimycotic solution 100× (Sigma-Aldrich) and 0.1% Gentamycine solution 50 mg/mL (Sigma-Aldrich). Immediately after cloning, the CRISPR-edited clones were cultured in EmbryoMax MEF Conditioned Media (Sigma-Aldrich) and, following adequate growth, subsequently cultured in complete DMEM. Hybridoma cells were maintained in 7 ml culture medium in T-25 flasks or 15 ml culture medium in T-75 flasks and subcultured every 48 or 72 h, depending on the culture conditions. Hybridoma cells were routinely tested for mycoplasma contamination and resulted negative throughout the study.

2.2.2 Hybridoma cells mRNA sequencing

Next Generation Sequencing (NGS) was carried out in order to characterize the VJC gene transcript set of the NS0 and 3E1 cell lines (Moreno et al., 2019). The 3E1 VJC gene set was used to design the donor construct and the sgRNAs. The mRNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions and quantified using a spectrophotometric method using Nanodrop system (Thermo Fisher Scientific). RNA was reverse transcribed into double-stranded cDNA using the Maxima H Minus Double–Stranded cDNA Synthesis Kit (Thermo Fisher Scientific), in accordance with manufacturer instructions. Oligo (dT)18 primers supplied by the kit were used, which selectively anneal to the 3'-end of poly(A)+ RNA, allowing the synthesis of cDNA only from poly(A)+ tailed mRNA. Complementary DNA was quantified using the Qubit 3.0 fluorometer (Thermo Fisher Scientific) using the dsDNA HS Kit (Thermo Fisher Scientific). Samples were used for DNA library preparation using the Nextera XT DNA Library Prep Kit (Illumina,

San Diego, CA, USA), according to the manufacturer's protocol. cDNA quantification was assessed using the Agilent DNA High Sensitivity chip assay (Agilent Technologies) and the Qubit dsDNA HS Kit (Thermo Fisher Scientific). Paired-end libraries were sequenced using Illumina V2 chemistry and the Illumina MiSeq platform. Sequencing reads were analyzed using Geneious software (ver. 11.1.2) via the resequencing approach. Briefly, reads were aligned to reference strains belonging to the V, J, and C regions of the mouse immunoglobulin light chain (Mus musculus, chromosome 6 - accession no. NC_000072.6). Once the specific V, J and C regions of the hybridoma were identified, a construct was designed in silico, and used as reference for a second resequencing step. Moreover, the construct was confirmed using a de novo assembly approach. Briefly, reads were assembled into contigs using Velvet software (ver. 1.2.10) (Zerbino & Birney, 2008) with the minimum contig length parameter set to 600 bp, and contigs were aligned to the VJC construct. The same method was carried out to verify the correct insertion of the GFP gene in the GFP-positive clones. The contigs were aligned to the consensus sequences obtained for each clone in order to confirm the VJC or VJ-GFP gene set and the correct coding frame of the final construct.

Optimization of the genome editing protocol, as described thereafter, was evaluated in the first experiments in terms of number of GFP positive cells and established taking into consideration the following parameters: number of transfected cells, µg of ssDNA, minus versus positive strand, transfection method, electroporation parameters.

2.2.3 Donor Construct for HR

We designed a DNA donor construct containing a multiple cloning site (MCS) between the two homology arms that flank the C gene region of the VJC gene set of hybridoma 3E1 line (HR-MCS), identified after the VJC gene set sequencing of the 3E1 cell line (Figure 4). The synthetic DNA fragment HR-MCS was ordered from Eurofins Genomics, and cloned into pUC18 to obtain pUC18-HR-MCS (Figure 5).

For the first experiment, the GFP gene was amplified via PCR using a BioRad T100™ Thermal Cycler, according to the following scheme:

Buffer coral 10x	5.0 µl	95°C 15 min	
mix dNTPs (10 mM each)	1.0 µl	35 cycle	95°C 1 min
Taq HS (Qiagen)	0.2 µl		67°C 1 min
Primer bamEgfps 10µM	1.0 µl		72°C 1 min
Primer xhoEgfpa 10µM	1.0 µl	72°C 10 min	
DNA template	5.0 µl		
H_2O	36.75µl		

The PCR product was visualised by electrophoresis on a 1.5% (w/v) agarose-gel, and then, it was column purified with PCR clean-up, gel extraction kit (Macherey-Nagel), according to the protocol of the manufacturers. The GFP gene was cloned into pUC18-HR-MCS between the two regions for homologous recombination, using BamHI and XhoI (Thermo Fisher Scientific) restriction sites, to obtain pUC18-HR-GFP (Figure 5).

For the second donor construct, pUC18-HR-GFP plasmid was restriction digested with BamHI (Thermo Fisher Scientific) and dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific). The BoHV1gE gene subunit encoding the glycoprotein ectodomain was amplified by PCR from a synthetic construct (pSec-BoHV1 cl9) developed in previous study (Bertolotti et al., 2013), using a BioRad T100™ Thermal Cycler, according to the following scheme:

Buffer coral 10x	5.0 µl	95°C 5 min	
mix dNTPs (10 mM each)	1.0 µl	35 cycles	95°C 1 min
Taq HS plus (Qiagen)	0.2 µl		55°C 40 sec
Primer BoHV1-Bam-f 10µM	1.0 µl		72°C 90 sec
Primer BoHV1-Bam-r 10µM	1.0 µl	72°C 10 min	
DNA template (pSec-BoHV1 cl9)	5.0 µl		
H_2O	36.75µl		

The PCR product was assessed by electrophoresis on a 1.5% (w/v) agarose-gel, and then, it was column purified with PCR clean-up, gel extraction kit (Macherey-Nagel), according to the protocol

of the manufacturers. The BoHV1gE gene was cloned into pUC18-HR-GFP between the first homologous recombination region and the GFP gene, using the BamHI (Thermo Fisher Scientific) restriction site, to obtain pUC18-HR-gE-GFP (Figure 5).

For the third donor construct, pUC18-HR-MCS was restriction digested with BamHI and XhoI (Thermo Fisher Scientific). The BoHV1gE gene subunit encoding the glycoprotein ectodomain was amplified by PCR using a BioRad T100™ Thermal Cycler, according to the following scheme:

<table>
<tr><td rowspan="7">Buffer coral 10x
mix dNTPs (10 mM each)
Taq HS plus (Qiagen)
Primer BoHV1-Bam-f 10µM
Primer BoHV1-Xho-r 10µM
DNA template (pSec-BoHV1 cl9)
H_2O</td><td rowspan="7">5.0 µl
1.0 µl
0.2 µl
1.0 µl
1.0 µl
5.0 µl
36.75µl</td><td colspan="2">95°C 5 min</td></tr>
<tr><td rowspan="3">35 cycles</td><td>95°C 1 min</td></tr>
<tr><td>55°C 40 sec</td></tr>
<tr><td>72°C 90 sec</td></tr>
<tr><td colspan="2">72°C 10 min</td></tr>
</table>

The PCR product was assessed by electrophoresis on a 1.5% (w/v) agarose-gel, and then, it was column purified with PCR clean-up, gel extraction kit (Macherey-Nagel), according to the protocol of the manufacturers. The BoHV1gE gene was cloned into pUC18-HR-MCS between the two homologous recombination regions to obtain pUC18-HR-gE (Figure 5).

All the ligation reactions were performed with T4 DNA ligase (Thermo Fisher Scientific). The correct insertions of all plasmids were assessed by Sanger sequencing. All the primer sequences are reported in Table 2. All the plasmids were amplified using *Escherichia coli* "Top10" competent cells, cultured as described in the section 3.2.1.

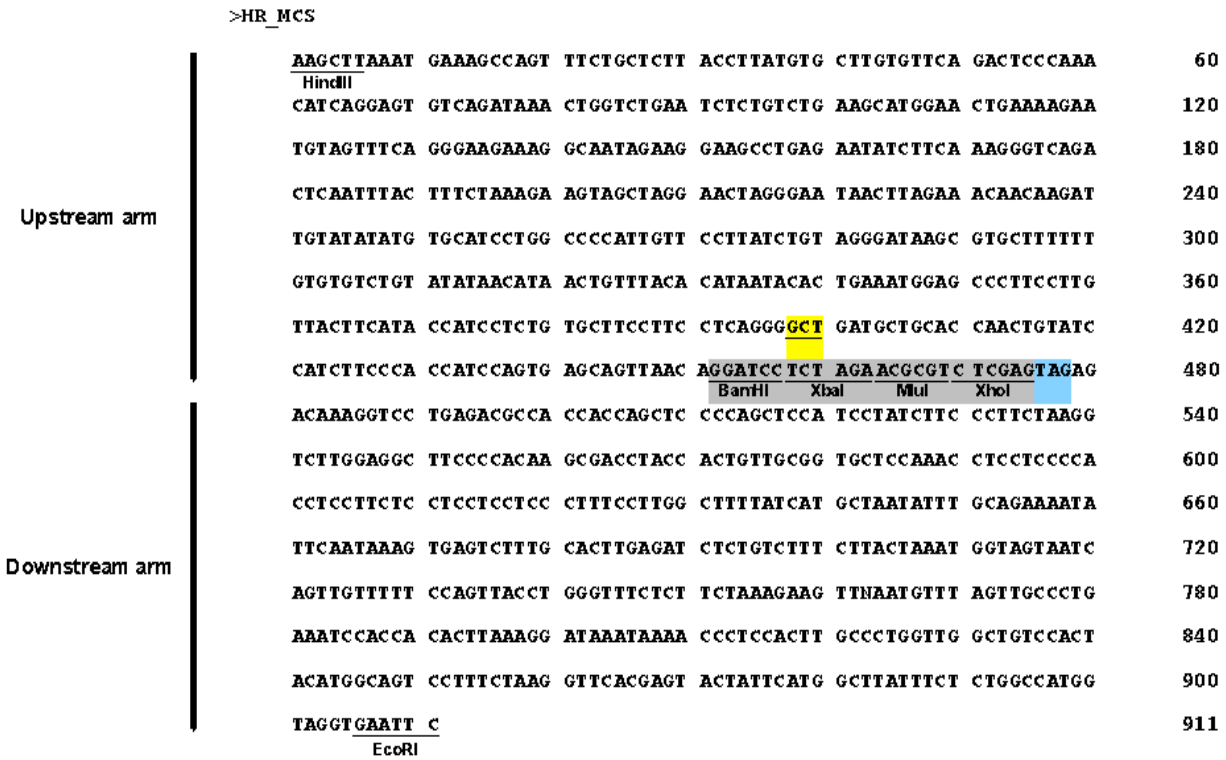

Figure 4: DNA sequence with a multiple cloning site (MCS) flanked by two arms for homologous recombination, upstream and downstream of the C gene of the VJC gene set of hybridoma 3E1 line. In grey, MSC (BamHI, XbaI, MluI and XhoI); in yellow (GCT), first amino acid of the C region; in blue (TAG), stop codon.

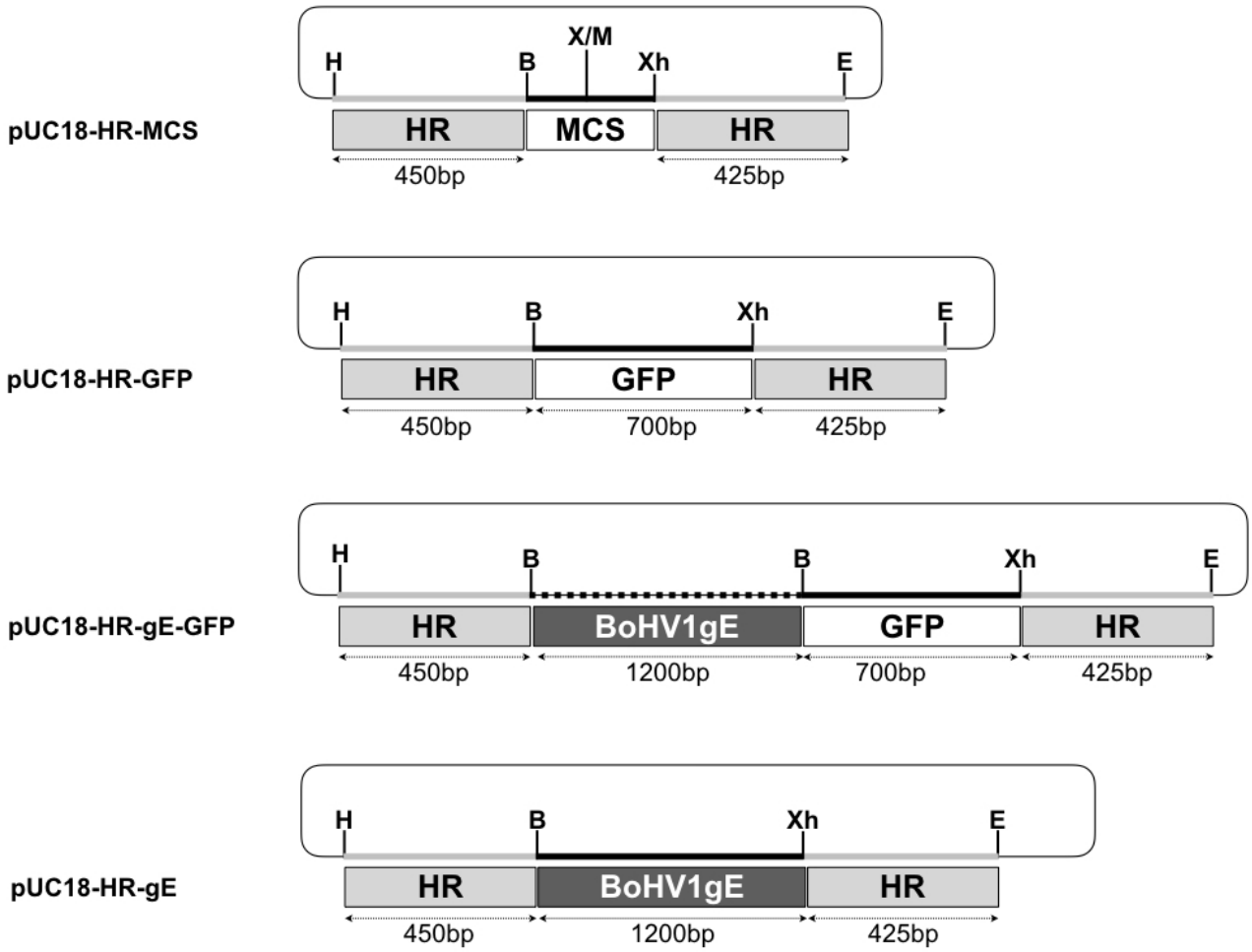

Figure 5: Schematic representation of the plasmids used in this study. Plasmid pUC18-HR-MCS containing a multiple cloning site (MCS) flanked by two homology arms for homologous recombination (HR). Plasmid pUC18-HR-GFP, containing the green fluorescent protein (GFP) coding sequence, derived from pUC18-HR-MCS. Plasmid pUC18-HR-gE-GFP, containing the Bovine Herpes Virus type 1 glycoprotein E (BoHV1gE) protein coding sequence fused with GFP, derived from pUC18-HR-GFP. Plasmid pUC18-HR-gE, containing the BoHV1gE protein coding sequence, derived from pUC18-HR-MCS. Numbers mentioned below the dotted arrows indicate the approximate size of the fragments. Restriction enzymes: B, BamHI; E, EcoRI ; H, HindIII ; M, MluI ; X, XbaI ; Xh, XhoI.

2.2.4 Single-stranded DNA production

Since the ssDNA donor is expected to increase HR efficiency, both ssDNAs (plus and minus strands) were obtained using the Guide-it Long ssDNA Strandase Kit (Takara Bio), according to the manufacturer's protocol.

Two different dsDNA PCR products were obtained using one regular PCR primer (I-f or I-r) and a second phosphorylated PCR primer (I-f-phos or I-r-phos) (Table 2), using specific plasmids as DNA templates, according to the following scheme:

PCR Reaction A:	PCR Reaction B:
50 µl - PrimeSTAR Max Premix (2X) 20–40 ng Template DNA 2 µl - Standard forward primer (40 µM) 2 µl - 5'-Phosphorylated reverse primer (40 µM) X µl - RNase Free Water	50 µl - PrimeSTAR Max Premix (2X) 20–40 ng Template DNA 2 µl - 5'-Phosphorylated forward primer (40 µM) 2 µl - Standard reverse primer(40 µM) X µl - RNase Free Water
Total volume 100 µl	Total volume 100 µl

The two PCR products were amplified using a BioRad T100™ Thermal Cycler, following the cycling conditions shown below:

35 cycles	98°C 10 sec
	55°C 5 sec
	72°C 5 sec/kb
4°C forever	

Each PCR reaction was analyzed on a 1.5% agarose gel, then column purified using the PCR Clean-up Gel Extraction Kit (Macherey-Nagel), according to the manufacturer's protocol. All PCR products were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) (dsDNA setting).

Each PCR product was independently subjected to two short and consecutive Strandase reactions to obtain ssDNA using a BioRad T100™ Thermal Cycler, according to the following scheme:

First reaction	
5–15 µg dsDNA substrate 5 µl Strandase A Buffer (10X) 5 µl Strandase Mix A X µl RNase Free Water	Cycling condition 37°C 5 min/kb 80°C 5 min 4°C until following step
Total volume 50 µl	

Second reaction	
50 µl Strandase reaction mixture (first reaction) 50 µl Strandase B Buffer (2X) 1 µl Strandase Mix B	Cycling conditions 37°C 5 min/kb 80°C 5 min 4°C until following step
Total volume 101 µl	

Only the phosphorylated strand, amplified using the phosphorylated primer, was subsequently degraded by treatment with two different enzymes: Strandase Mix A and Strandase Mix B. Finally, 5 µl of each ssDNA sample was analyzed on a 1.5% agarose gel, including 100 ng of the dsDNA substrate in a separate lane as control. All products were purified and subsequently quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) (ssDNA setting).

The ssDNA donor repair construct with the GFP gene flanked by two regions for homologous recombination (HR-GFP), the ssDNA construct with the BoHV1gE gene fused in frame with the GFP gene (HR-gE-GFP), and the ssDNA construct with the BoHV1gE gene (HR-gE) were obtained using the plasmids pUC18-HR-GFP, pUC18-HR-gE-GFP and pUC18-HR-gE (Figure 5), respectively, as DNA templates in the PCR reaction.

2.2.5 Single guide RNA and Cas9/sgRNA complex

The sgRNAs were obtained using a Guide-it sgRNA In Vitro Transcription and Screening Systems Kit (Takara), according to the manufacturer's protocol.

A forward PCR primer was designed and had to contain:

- a T7 promoter sequence at its 5' end;
- a transcription initiation site (0–2 guanine (G) residues);
- a 20-nucleotide sgRNA target sequence;
- the Guide-it Scaffold Template-annealing sequence at its 3'end.

The sgRNA template was amplified by PCR, using a BioRad T100™ Thermal Cycler, according to the following scheme:

PrimeSTAR Max Premix (2X) Guide-it Scaffold Template Forward primer (10 µM) RNase Free Water	12.5 µl 1.0 µl 0.5 µl 11.0 µl	33 cycles	98°C 10 sec 68°C 10 sec
Total volume 50 µl		4°C forever	

The two sgRNA, named sgRNA-tag1 and sgRNA-tag2, were designed in silico according to the guidelines described in the Guide-it Complete sgRNA Screening System Kit (Takara Bio) in order to drive the two DSBs, located up- and downstream of the antibody light constant region of the 3E1 genome. They were used at the same time in order to obtain two DSBs simultaneously. All the primers designed and used to obtain the sgRNAs are listed in Table 2.

After the PCR run, 5 µl of each sample was analyzed on a 1.5% agarose gel in order to visualize a single band of 130 bp. The PCR product was used directly, without purification, as template for the in vitro transcription (IVT) reaction, using a BioRad T100™ Thermal Cycler, according to the following scheme:

sgRNA PCR template Guide-it In Vitro Transcription Buffer Guide-it T7 Polymerase Mix RNase Free Water	5.0 µl 7.0 µl 3.0 µl 5.0 µl	37°C 4 hours 4°C forever
Total volume 20 µl	20 µl	
Recombinant DNase I (RNase-Free)	2.0 µl	37°C 15 minutes 4°C forever
Total volume	22 µl	

Then, the product of the IVT reaction was column purified using the Guide-it IVT RNA Clean-Up Kit (Takara), according to the manufacturer's protocol. Each product was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) (RNA setting)

2.2.6 Cas9-sgRNA COMPLEX

Cas9 (Recombinant wild-type *Streptococcus pyogenes* Cas9 nuclease - Takara) and the sgRNAs were combined at a mass ratio of 5:1 (using the same buffer as used to resuspend the cells) to form RNP complexes. The reagents were mixed by gently pipetting the solution up and down, then incubated in a BioRad T100™ Thermal Cycler, according to the following program:

- 37°C 5 min → then 4°C until the next step.

Table 2: List of primers used in this study. I-F, I-R, I-F-phos and I-R-phos used for the ssDNA donor DNA production; bamEGFPs and xhoEGFPa used for the GFP gene PCR amplification; BoHV1-Bam-f and BoHV1-Bam-r used for the BoHV1gE gene PCR amplification; I-sg1 and I-sg2 used for the production of the sgRNA-tag1 and sgRNA-tag2 respectively. Restriction sites are underlined.

Name	Primer sequence (5' to 3')
I-F	AAATGAAAGCCAGTTTCTGCTCTTAC
I-R	ACCTACCATGGCCAGAGAAATAAGC
I-F-phos	[PHOS]AAATGAAAGCCAGTTTCTGCTCTTAC
I-R-phos	[PHOS]ACCTACCATGGCCAGAGAAATAAGC
bamEGFPs	TTGGATCCGTGAGCAAGGGCGAGGAGCTGTTC
xhoEGFPa	TTCTCGAGCTTGTACAGCTCGTCCATGCCGAG
BoHV1-Bam-f	TTGGATCCAAACCGGCCACCGAGACACCCG
BoHV1-Bam-r	TTGGATCCCTCGGAGGTCAAGGGTGGCCC
BoHV1-Xho-r	TTCTCGAGCTCGGAGGTCAAGGGTGGCCC
I-sg1	CCTCTAATACGACTCACTATAGGTCCAGTGAGCAGTTAACATCGTTTAAGAGCTATGC
I-sg2	CCTCTAATACGACTCACTATAGGAATGAGTGTTAGAGACAAGTTTAAGAGCTATGC

2.2.7 Cell line transfection

The transfection was performed by electroporation using Cell Line Nucleofector Kits C and L (Lonza) with a Nucleofector II device (Amaxa), according to the protocol proposed by the manufacturer. Briefly, $2x10^6$ hybridoma cells (line 3E1) were harvested by centrifugation at 90 × g at room temperature for 10 minutes and culture medium removed by aspiration. The pellet was gently resuspended in electroporation buffer, mixed with the Cas9/sgRNA complexes and 1 μg of single-stranded donor DNA (plus strand) (100 μl total volume) and electroporated using the program A-020. Following electroporation, transfected cells were transferred into a 6-well plate containing 2 ml fresh DMEM without antibiotic-antimycotic solution and incubated for 72 hours.

2.2.8 Isolation of the cell line

GFP-positive cells were isolated using the FACSAria IIIu cell sorter (Beckton Dickinson). In the first experiment, single cells were sorted into the wells of multiple 96-well plates containing 200 μl of conditioned medium. After 15 days, the clones were analyzed via inverted fluorescence microscopy and the GFP-positive clones were scaled up into 24-well and then 6-well plates. At this time, fluorimetric analysis of the medium and a NGS sequencing of a subset of GFP positive clones were carried out. The clones were propagated for several passages and analyzed at each passage by flow cytometry to verify the percentage of GFP-positive cells.

In the second experiment, where the ssHR-gE-GFP donor sequence was used, the cells were first sorted to obtain a homogeneous GFP-positive population delivered and grown in a single well of a multiple 6-well plate containing 2 ml conditioned medium. Then, GFP-positive hybridoma clones were singularly cloned by limiting dilution in a 96-well plate and grown in 200 μl conditioned medium. After 15 days, the clones were analyzed using an inverted fluorescence microscope and the GFP positive clones were scaled up into a 24-well plate. At this stage, an ELISA sandwich test and a Western blot using anti-gE specific mAbs were carried out on replica wells, following standard procedures (Borrè et al., 2004), on both the supernatant and the cell lysate to verify that the expressed protein was successfully secreted.

In the third experiment, where the ssHR-gE donor sequence was used, GFP-negative cells were isolated using the FACSAria IIIu cell sorter (Beckton Dickinson). In this experiment, single cells

were sorted into the wells of multiple 96-well plates containing 200 µl of conditioned medium. After 15 days, the clones were analyzed via inverted fluorescence microscopy. At this stage, an ELISA sandwich test using anti-gE specific mAbs were carried out on replica wells, following standard procedures (Borrè et al., 2004), on both the supernatant and the cell lysate to verify the protein expression.

2.2.9 Fluorimetric analysis

In order to verify the secretion of GFP, the growth medium of each clone was analyzed by fluorescence spectroscopy using the Qubit 3.0 fluorometer (Thermo Fischer Scientific), according to the protocol suggested by the manufacturer. Briefly, 200 µl of growth medium was collected at least 24 hours after cell splitting and analyzed using the Qubit fluorometer mode, with the blue excitation light (470 nm) and the reading fluorescence in the green emission channel (510-580 nm). A mock sample was used to assess the background fluorescence of the medium.

2.2.10 ELISA

Plates were coated with the capture monoclonal antibody (Egyed et al., 1992) by incubation overnight at 4°C, and then blocked with 2.5% bovine casein. The samples were diluted 1:2 in a PBS-Triton 1% solution, added to the wells, and the plates were then incubated for 1 h at room temperature. Following a washing step, a peroxidase-labelled secondary monoclonal antibody (Egyed et al., 1992) diluted in saline buffer was added, and the plates were incubated for 1 h at room temperature. After the final washing step, a solution of 3,3′,5,5′-tetramethylbenzidine (TMB) was used to develop the colour reaction, which was stopped after 15 minutes by the addition of sulfuric acid stop solution. Finally, the signal was measured by reading the absorbance in a microplate spectrophotometer at 450 nm.

In addition, an indirect ELISA using a set of well characterized bovine sera was carried out to evaluate antigenic properties and the diagnostic potential of the recombinant gE.

2.2.11 Western Blot

The samples were subjected to sodium dodecyl sulphate polyacrylamide (10%) gel electrophoresis (SDS-PAGE), performed on a Protean Tetra Cell (Bio-Rad) according to the manufacturer's instructions. Finally, the gel was stained using PageBlue^{TM} Protein Staining Solution (Fermentas), or otherwise subjected to western immunoblotting.

The gel was transferred to a 0.45 µm nitrocellulose blotting membrane (Sigma-Aldrich) and incubated overnight at 30 V and 4°C, or for 1 hour at 100V at room temperature. Afterwards, the membrane was blocked for 2 hours at room temperature using 2.5% bovine casein diluted in TBS 1X. After a washing step, the membrane was incubated with a horseradish peroxidase (HRP)-labelled anti-gE mAb, (Borrè et al., 2004) diluted in Tris-buffered saline (TBS) 1X, for 2 hours at 4°C. Finally, the membrane was developed using DAB.

2.2.12 BoHV1gE quantification

In order to quantify protein secretion, gE concentration was roughly estimated using serial twofold dilution of three clone's growth medium coated onto a solid surface and probed with anti-gE specific mAb by means of indirect ELISA. A Known concentration of serially diluted recombinant gE protein, expressed in a prokaryotic system and known to be reactive against the same mAb, were used as positive control and to obtain a standard curve. A sample of the original 3E1 growth medium was used as negative control.

2.3 Results and discussion

Recent studies successfully demonstrated the use of the CRISPR/Cas9 system combined with HDR to edit the genome of hybridomas (Cheong et al., 2016; Pogson et al., 2016; Khoshnejad et al., 2018; Parola et al., 2018; Parola et al., 2019; van der Schoot et al., 2019).

In the present study, we evaluated the feasibility of using the Cas9 RNP in combination with single strand donor DNA to obtain a stable hybridoma cell line able to express and secrete protein of diagnostic interest into the culture medium. The hybridoma cell line 3E1 was chosen for use in the present study because of the high production yield (more than 1mg/ml) of the specific mAb when grown in a high-density stationary culture system (CELLine bioreactor flask) (Moreno et al., 2019).

In this pilot study, we focused on the kappa light chain constant region, replacing it with a gene of interest and exploiting the original secretion signal, essential for the secretion of recombinant protein into the medium. We used the RNP complex, due to its notable advantages for genome engineering compared with other CRISPR/Cas9 delivery methods. The RNP complex offers fast editing and it has been demonstrated to be degraded in 24 hours, in contrast with the continuous expression of the Cas9 protein from delivered coding plasmid, which may persist for several days (Kim et al., 2014). This limited activity of the RNP complex reduced off-target mutations and any toxic effects upon the cells.

2.3.1 mRNA sequencing

Sequence analyses were carried out to characterize the light chain gene set in NS0 (fusion partner cell line) and 3E1 cell lines (Moreno et al., 2019). A summary of the results is reported in Table 3. Even if a variable number of reads was obtained in each experiment, the results provided the needed support for the correct identification of the more expressed VJC gene sets.

The 3E1 VJC gene set was used to design the donor construct HR-MCS that contains two homology arms that flank the C gene region of the VJC gene set. The first homology arm includes an intron upstream of the C gene region (Figure 6-A).

Interestingly, the sequencing data from the NS0 cell line revealed the presence of the V3.12/J2*/C gene set, harboring a stop codon in the J2 region and representing an inactive light chain. The 3E1 cell line showed the co-presence of two light chains. Some reads (36.3%) assembled a V3.12/J2*/C gene set but the majority of reads (63.7%) described a functionally active V12.41/J4/C gene set. The latter sequence was identified in the murine chromosome 6.

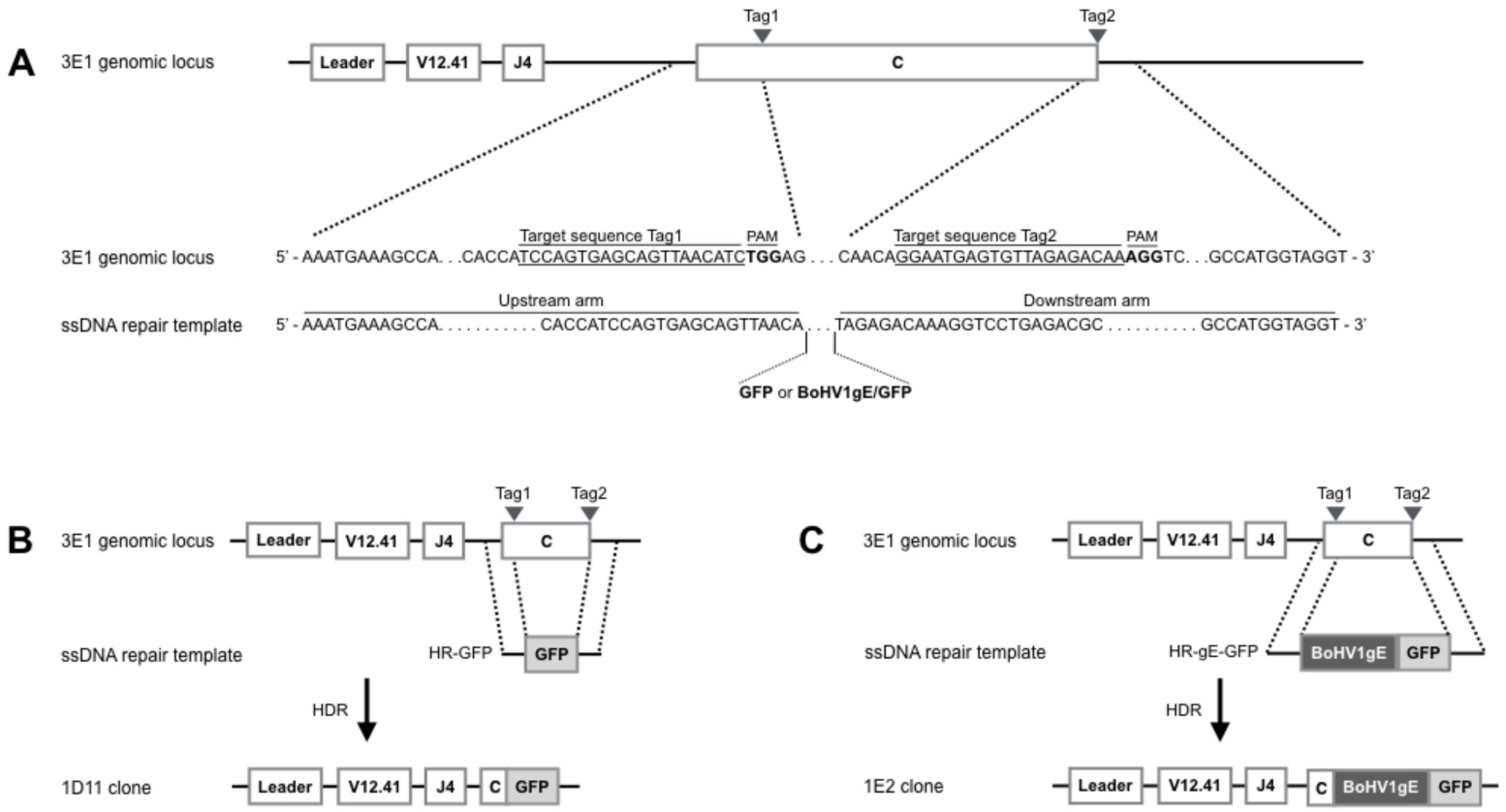

Figure 6: A schematic representation of HDR after two DSBs in the kappa light chain constant region. The two Cas9/sgRNA complexes (Tag1 and Tag2) made two DSBs upstream and downstream of the light chain C region (black triangle). (**A**) Single-stranded donor DNAs are used in combination with Cas9/sgRNA complexes for HDR-mediated editing at the target locus. The DSBs were repaired by HDR using: the HR-GFP single-stranded donor, (**B**) or the HR-gE-GFP single-stranded donor (**C**) DNA. BoHV1gE: Bovine Herpes Virus 1 Glycoprotein E gene; C: constant gene segment; GFP: Green Fluorescent Protein gene; J4: joining gene segment; Leader: leader peptide that enables protein secretion, and is subsequently cleaved after translation; V12-41: variable gene segment.

Table 3: Summary of NGS sequencing results. The number of sequencing reads mapping to the different gene set of light chain immunoglobulin. Average coverage obtained for each clone is reported.

Hybridoma	Reads number	V3.12/J2*/C		V12.41/J4/C		V12.41/J4/C-GFP	
		Mean coverage	(%)	Mean coverage	(%)	Mean coverage	(%)
NS0	2 648 666	9 722 (1872x)	100 %	/	/	/	/
3E1	651 144	340 (67x)	36.3%	597 (119x)	63.7%	/	/
1D11	3 882 734	12 497 (3693x)	32.1%	/	/	26 493 (4922x)	67.9%
1D2	27 336 352	4 268 (56x)	49.9%	/	/	4 292 (253x)	50.1%
2B2	3 753 530	7 468 (2221x)	66.1%	/	/	3 822 (782x)	33.9%
2C10	2 608 540	7 156 (1502x)	35.8%	/	/	12 843 (1.558x)	64.2%
2D4	2 447 332	10 568 (2.220x)	99.4%	/	/	65 (8x)	0.60%

2.3.2 GFP expression

Firstly, with the aim of evaluating the efficiency of our method, we used the donor DNA bearing the GFP gene (HR-GFP) (Figure 6-B).

A total of $2x10^6$ 3E1 cells were co-transfected with the Cas9/sgRNA complexes (obtained using both sgRNA-Tag1 and sgRNA-Tag2) and the ssDNA donor repair construct HR-GFP (Figure 7). Seventy-two hours post transfection, approx. 150 GFP-positive cells were sorted by FACS analysis and singularly transferred into 96-well plates and cultured in conditioned medium. Following 3 weeks of incubation, we obtained 10 GFP-positive clones. The correct insertion of the GFP gene between the two homologous recombination zones was checked by NGS sequencing in 5 clones, named 1D11, 1D2, 2B2, 2C10 and 2D4. The number of sequencing reads obtained for each clone, as well as for the parental 3E1 hybridoma and the NS0 fusion partner cell line, is reported in Table 3. Sequence analysis showed the presence of two VJC gene sets for each clone: the functionally active V12.41/J4/GFP and the non-codifying V3.12/J2*/C gene set (also present in the NS0 fusion partner). Notably, the V12.41/J4/C gene set, found in the original 3E1clone, was never recorded.

We analyzed the growth medium of the clones by fluorescence spectroscopy to confirm the secretion of GFP into the medium. Clone 1D11 produced the highest fluorescence value (Figure 8).

Considering the results obtained by NGS sequencing and the fluorescence spectroscopy data for the medium of each clone, clone 1D11 was selected for further investigation. In particular, NGS data analysis for clone 1D11 confirmed the substitution of part of the C gene with the GFP counterpart. Moreover, no contigs harboring the GFP gene and mapping to other regions of the murine genome (chromosome 6) were recorded, confirming the absence of off-target insertions of the GFP gene.

An indirect ELISA with a semi-purified FluD antigen coated onto the solid phase was performed to analyze the reactivity of anti-FluD mAb of the original 3E1 hybridoma. As expected, ELISA reactivity was completely abolished in the 1D11 line, compared with the original line 3E1, confirming the absence of a functional antibody molecule (Figure 9).

Furthermore, clone 1D11 was propagated for several passages to investigate whether the percentage of GFP-positive cells remained constant. Flow cytometry analysis confirmed that the number of GFP-positive cells was constantly higher than 95% for up to 8 passages. Surprisingly, we never obtained 100% GFP-positive cells by FACS analysis, even after sequential re-sorting purification. Although this aspect needs to be evaluated over a longer time period, it is probable that the small percentage of GFP-negative cells did not significantly affect the expressed protein

concentration. In a recent study, following CRISPR/HDR-hybridoma genome editing, van der Schoot and colleagues did not observe any reduction in antibody production over a period of 1-3 years (van der Schoot et al., 2019).

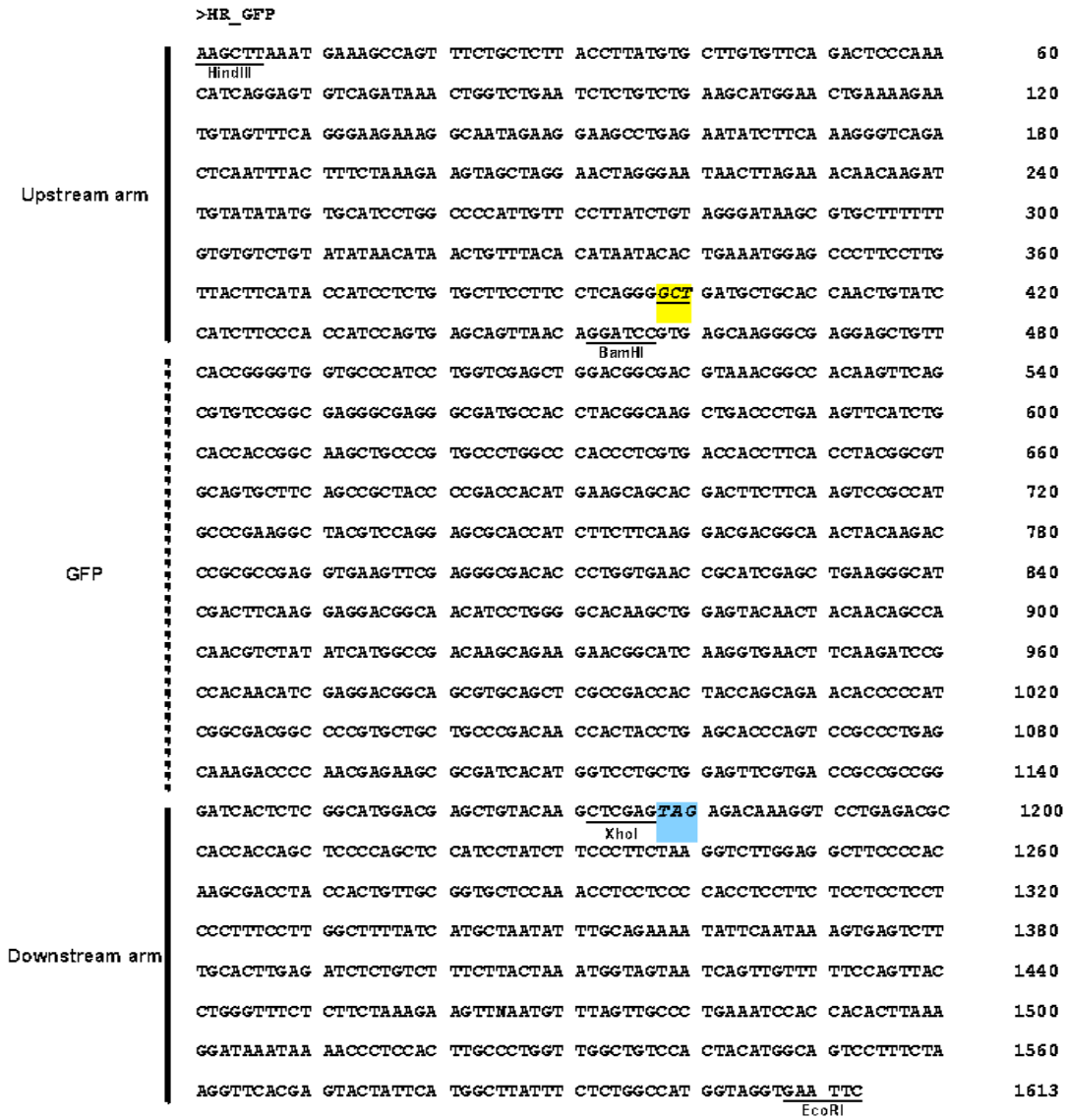

Figure 7: Sequence of the DNA donor repair HR-GFP containing green fluorescent protein (GFP) coding sequence flanked by two arms for homologous recombination, upstream and downstream of the C gene of the VJC gene set of hybridoma 3E1 line. In yellow (GCT), first amino acid of the C region; in blue (TAG), stop codon.

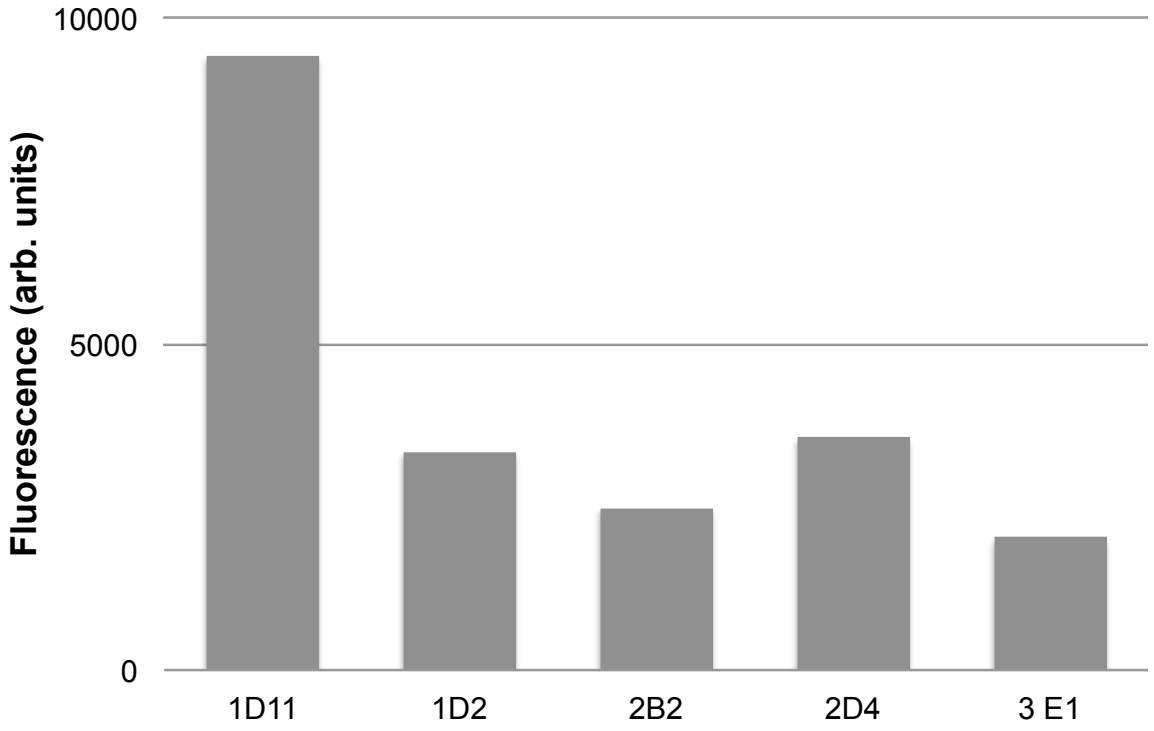

Figure 8: Fluorescence spectroscopy analysis of the growth medium of clones 1D11, 1D2, 2B2, 2D4, and original 3E1 hybridoma cell line.

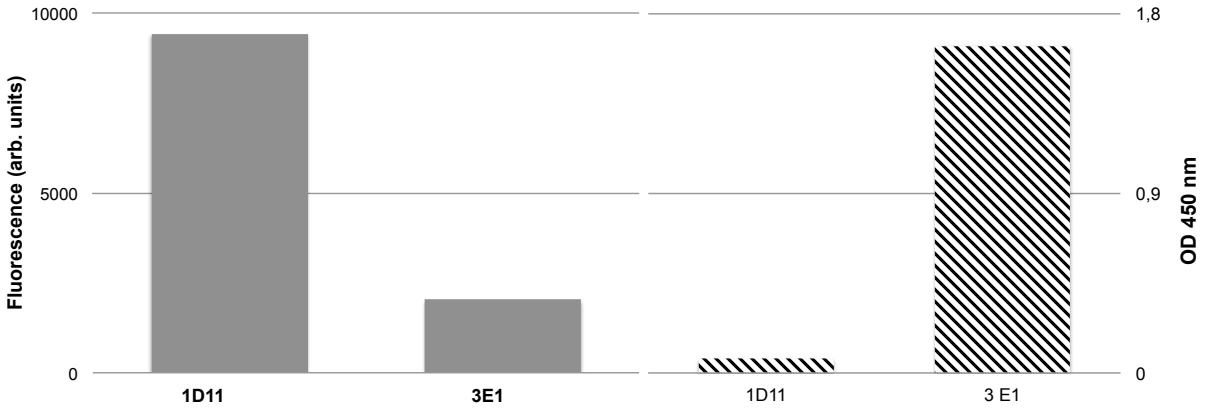

Figure 9: Analysis of the 3E1 and 1D11 growth medium. On the left y axis, fluorescence spectroscopy results; on the right y axis, ELISA sandwich results. ELISA sandwich analyses were performed using anti-FluD mAb of the original 3E1 line.

2.3.3 BoHV1 glycoprotein E expression

Following the successful gene editing obtained with the line 1D11, we focused on a different construct to express a protein of diagnostic interest. We attempted to express the BoHV1 glycoprotein E in fusion with GFP. We transfected $2x10^6$ cells of the 3E1 hybridoma cell line using the same Cas9/sgRNA complexes employed in the first experiment and with the ssDNA donor repair construct bearing the BoHV1gE gene fused in frame with the GFP gene (HR-gE-GFP) (Figure 6-C). Seventy-two hours post-transfection, approx. 400 GFP-positive cells were isolated via FACS analysis and cultured in conditioned medium into a single well of a 6-well plate. After one week of incubation (Figure 10), 200 vital cells were retrieved and, after dilution, single cells/well were seeded in 96-well plates. After 3 weeks of incubation, we obtained 11 GFP-positive clones, analyzed using an inverted fluorescence microscope.

We observed a phototoxic effect in the hybridoma cells following UV light exposure using both the HR-GFP and HR-gE-GFP donors; this photobleaching effect is strongly dependent on the redox environment and it appeared to make those cells more sensitive than other cell lines (Greenbaum et al., 2000; Icha et al., 2017). As a result, in the last experiment, using the HR-gE-GFP donor, it was necessary to minimize the analysis of the transfected cells by fluorescence microscopy to ensure a higher viability rate in the single cell cloning procedure.

Single cell sorting, applied in the first experiment was also abandoned in gE-GFP editing and replaced by single cell cloning using a limiting dilution approach which ensured the best cell viability. In fact, this method damages and stresses cells much less compared with single cell sorting. The 11 GFP positive clones with the highest viability and the fastest cell growth were chosen for further analysis to verify the capability of the clones to express and secrete the protein into the medium.

After the isolation of GFP-positive clones, we performed several tests to verify the capability of the clones to express and secrete the protein into the medium.

We characterized all 11 clones using immunological methods. First, the culture medium and the lysate of each clone were analyzed by a sandwich ELISA, performed with a capture and a horseradish peroxidase (HRP)-labelled anti-gE mAb recognising two different epitopes (Borrè et al., 2004), and 10 out of 11 clones gave a positive result (Figure 11-A), confirming the expression and the secretion of the gE protein into the medium.

Of the 10 positive clones that gave a positive ELISA result, the one with the highest viability and the fastest cell growth (named 1E2) was further characterized by Western Blot analysis using an HRP-labeled mAb known to recognize a gE linear epitope (Figure 12) (Borrè et al., 2004). We

confirmed the expression of a protein band of about 90 KDa, corresponding to the full expression of the predicted polyprotein V12.41/gEectodomain/GFP, in clone 1E2.

It should be noted that both the capture and detection mAbs were directed against conformational epitopes, known to be of diagnostic relevance.

In addition, an indirect ELISA was performed using bovine sera (four from naturally infected and three for marker vaccinated animals) and the antigen/antibody complex detected using HRP-labelled anti-bovine IgG (Figure 11-B). This finding provided evidence that the actual gene set orientation may confer the correct folding of gE, thereby preserving reactivity of conformational epitopes.

Finally, a standard curve created in ELISA using serial dilution of a known positive control showed excellent linearity (R^2 = 0.999) and allowed to quantify the expressed protein (Figure 13). The gE concentration in the growth medium of the three different positive clones with the fastest cell growth (including the clone 1E2) was in the range of 70-100 µg/ml in three different positive clones.

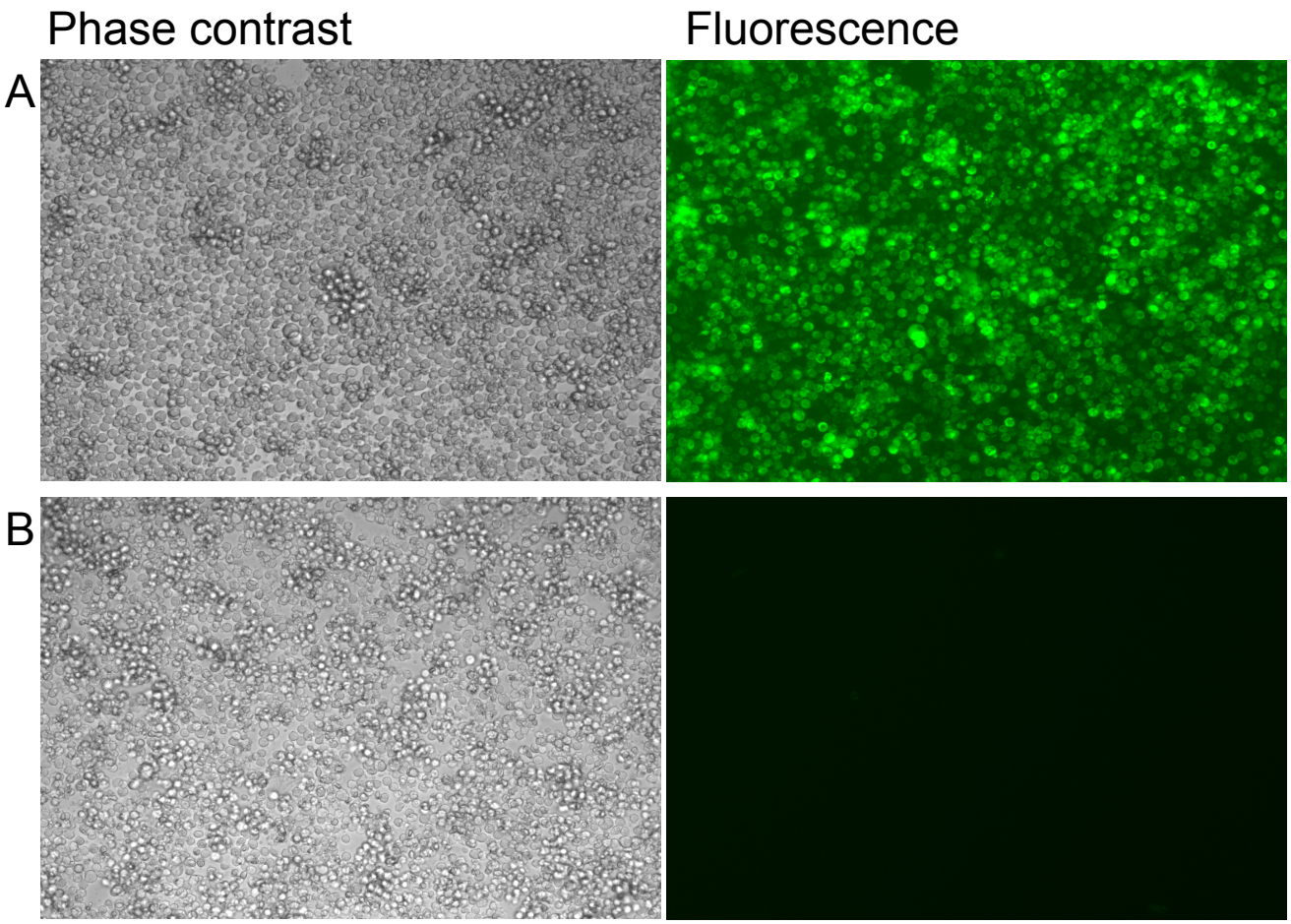

Figure 10: Fluorescence microscopy analysis of (A) GFP positive cells post sorting transfected with Cas9/sgRNA complex and ssDNA donor repair construct HR-gE-GFP, (B) GFP negative cells. Images were acquired with a 10× objective.

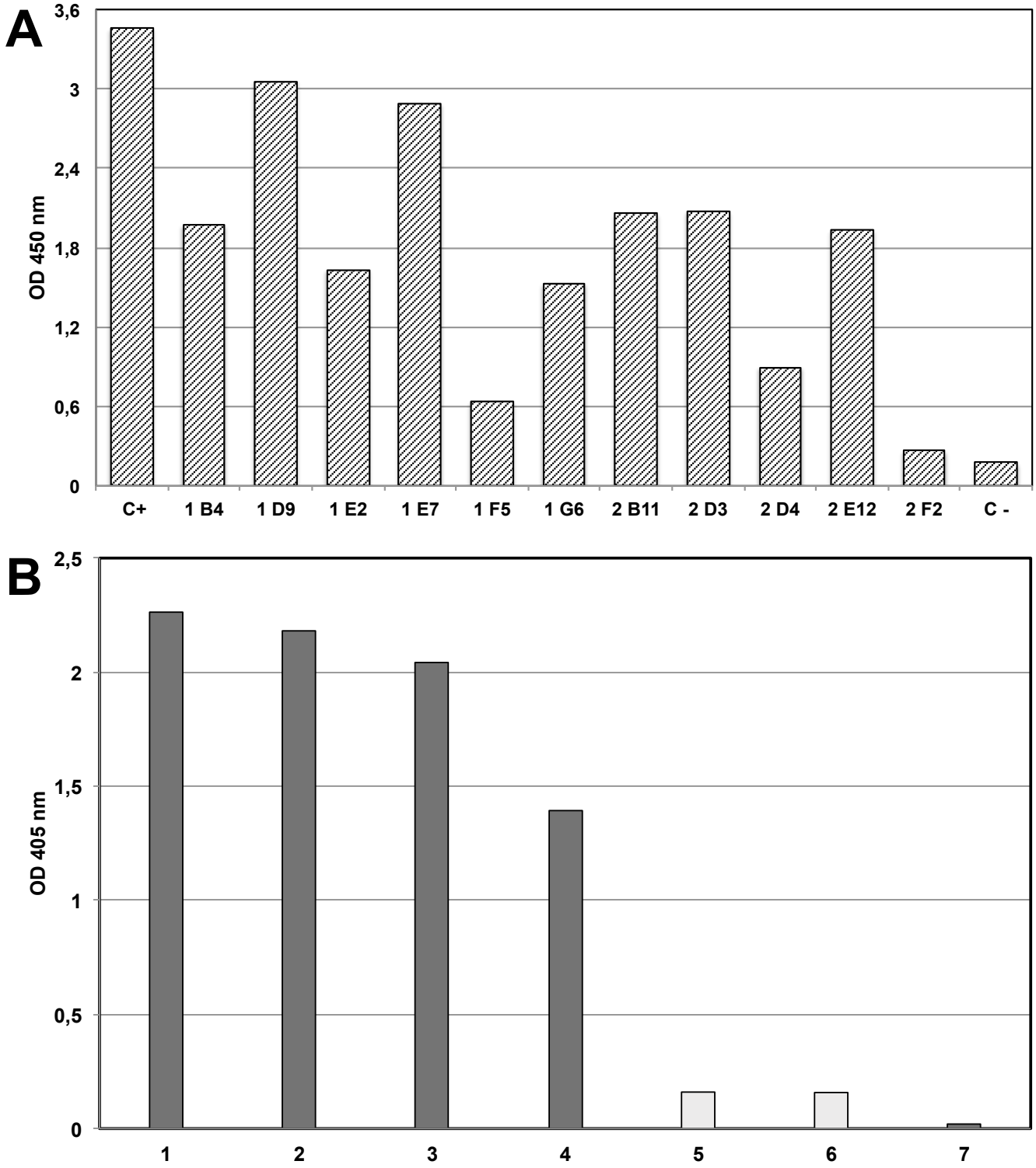

Figure 11: (See legend on next page).

Figure 11: A) ELISA sandwich results of the growth medium of the clones obtained after the transfection using the Cas9/sgRNA complexes and the ssDNA donor repair construct HR-gE-GFP. The analysis was performed using a capture and a horseradish peroxidase (HRP)-labelled anti-gE mAb (Borrè et al., 2004). All the clones, except the clone 2F2, gave a positive result. C+, positive control (recombinant BoHV1gE protein); C-, negative control sample (3E1 clone growth medium). **B)** Indirect ELISA results of bovine sera from naturally infected (1, 2, 3 and 4) and from marker vaccinated gE negative (5, 6 and 7) animals. The antigen/antibody complex was detected using HRP-labelled anti-bovine IgG.

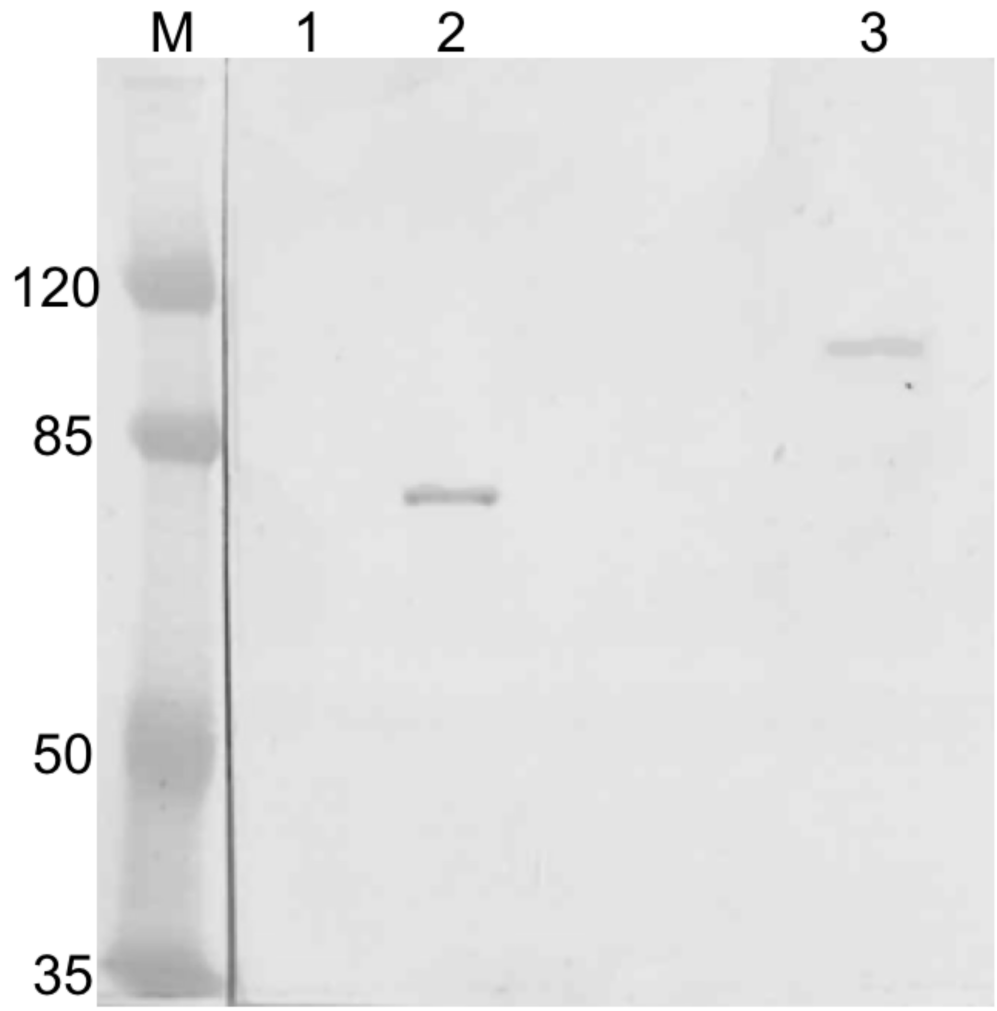

Figure 12: Western blot analysis using anti gE HRP-labelled mAb. Lane M: prestained molecular weight (sizes are expressed in kDa); lane 1: negative control sample (3E1 clone pellet); lane 2: recombinant BoHV1gE protein; lane 3: 1E2 clone pellet.

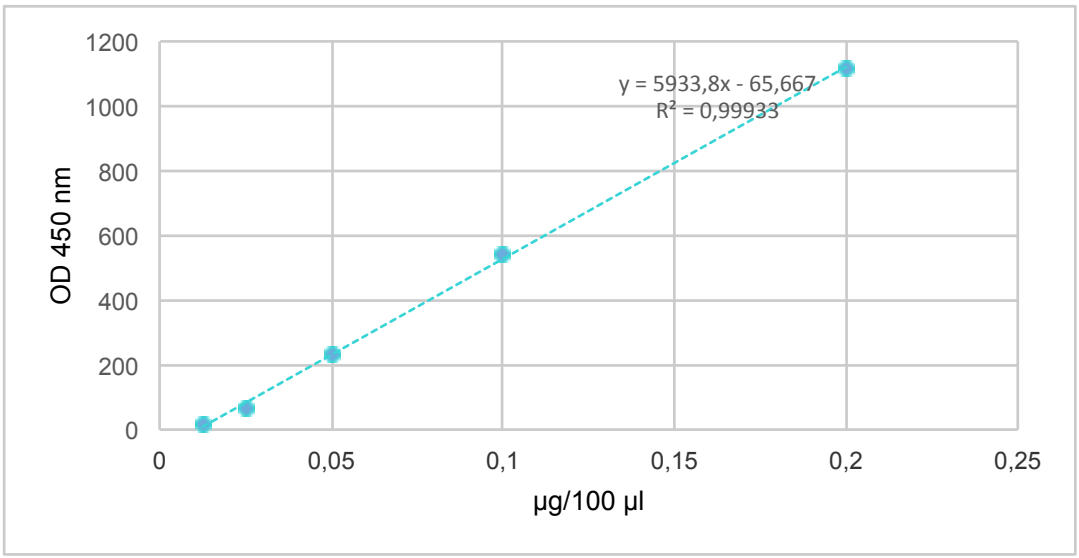

Figure 13: Standard curve (linearity: R^2 = 0.999) created using serial dilutions of a recombinant BoHV1gE (Bovine Herpesvirus 1 glycoprotein E) protein expressed in prokaryotic system and known to be reactive against the same mAb.

2.4 Conclusions

In the long and complex process of developing new diagnostic tools for disease control, the production of a protein of diagnostic interest provides a general starting point. Recombinant protein expression technology can be used to investigate the structure, function and interactions of this protein, as well as enzyme, antibody and vaccine production. In these latter applications, a higher amount of protein is required, and the inclusion of correct post-translational modifications are often fundamental. For this reason, protein expression systems based on mammalian cells are preferred to those using bacteria, yeast or insect cells, because correct post-translational modifications are more likely.

Transient mammalian expression systems are generally used for small-scale productions since protein expression is limited to just few days, but they are not recommended when highly reproducible results are required (Wurm, 2004; Bollin et al., 2011). Indeed, a stable mammalian protein expression system requires the integration of the gene of interest into the genome, which generally occurs in a random manner, and although stable over time results in very low product yields (Büssow, 2015).

In this research project, we explored the use of the CRISPR-Cas9 technology, due to its efficiency, specificity and capacity to avoid random integration, to genome edit a cell line (hybridoma) in a precise region with a high level of transcriptional activity (the immunoglobulin light chain). The aim was to develop a stable system for high-yield protein expression; achieving this aim would constitute a significant advancement in the development of diagnostic tools for veterinary applications.

In particular, this study demonstrates the efficient use of the CRISPR/Cas9 system (Cas9/sgRNA complex) in combination with single-stranded donor DNA to genome edit the hybridoma cell line 3E1, developed in a previous study (Moreno et al., 2019). This cell line was chosen for use in the present study because of the high production yield (more than 1mg/ml) of the specific mAb (mAb-FluD) when grown in a high-density stationary culture system (CELLine bioreactor flask).

The engineered clones 1D11 and 1E2, expressing GFP and BoHV1gE/GFP fusion protein, respectively, showed high viability, fast cell growth and were able to express and secrete the protein of interest into the culture medium.

The NGS sequencing of the clone 1D11 confirmed the correct insertion of the GFP gene between the two homologous recombination zones. The analysis also confirmed the absence of off-

target insertions of the GFP gene, because no contigs harboring the GFP gene were recorded at other regions of the murine genome.

The BoHV1gE/GFP expression by the 1E2 clone was confirmed, not only by ELISA, but also via Western Blot analysis using an HRP-labeled mAb (Borrè et al., 2004). It should be underlined that both the capture and detection mAbs used were directed against conformational epitopes known to be of diagnostic relevance. Furthermore, this finding was further supplied via indirect ELISA performed using bovine sera (four from naturally infected and three for marker vaccinated animals), confirming that the actual set gene orientation probably confers the correct folding of gE, and therefore the reactivity of its conformational epitopes.

Finally, we estimated the concentration of BoHV1gE in the growth medium, found to range between 70–100 µg/ml in three different positive clones, including 1E2.

In these experiments, the low efficiency of HDR was efficiently counteracted through the use of a fluorescence-based sorting procedure, which provided an easy and fast selection method.

In the future, the system could be further improved by genome editing GFP-expressing cells in order to replace GFP with a protein of interest. Thereafter, the GFP-negative cells will only express the protein of interest, instead of GFP, and they can be isolated by means of fluorescence-based sorting method. In this research, CRISPR-Cas9 editing was used to replace, in a single attempt, the GFP gene inserted into the engineered clone 1D11 (demonstrated to be genetically stable) with the gene encoding BoHV1gE. Seventy GFP-negative cells were single cell sorted, cultured in conditioned medium for about 15 days, and then analyzed by ELISA. However, all clones scored negative for gE expression (data not shown).

Although the results obtained with the 1D11 line demonstrated stable genome editing, the small, but persistent, percentage of cells negative for GFP expression strongly limited the feasibility of using this clone for further genome editing in combination with the fluorescence-based sorting procedure. Indeed, even after sequential re-sorting purification, flow cytometry analysis of the 1D11 line showed that the number of GFP-positive cells was constantly higher than 95%, but never 100%. This level of 5% GFP-negative cells was clearly well above the percentage of CRISPR-Cas9 edited cells achieved in our hands (about 0.075%, ratio 66:1). However, we have yet to analyze the transcriptome of the GFP-negative clones derived from the 1D11 cell line and future analysis will be crucial to improve our understanding of the underlying cause of this result.

In this study, we focussed on BoHV1 glycoprotein E since this recombinant protein had already been successfully used in assay format for an IBR surveillance program in a region of north-west Italy (Piedmont) (Bertolotti et al., 2015; Muratore et al., 2017; Colitti et al., 2018). Consequently, the development of a stable system for the high-yield expression of BoHV1gE would be

important for the further development of tools for IBR disease control. However, this system could be applied to any protein of diagnostic interest whose gene sequence is known. In the future, an interesting development may be the expression of different proteins to exploit in the field of veterinary or human infectious disease diagnostics.

Other further developments will involve V gene replacement with a protein-based affinity tag by means of a larger gene deletion. Indeed, many protein-based fusion tags have been developed to improve the detection, purification and solubility enhancement of the protein (Loughran & Walls, 2017). The result will be the expression of a protein fused to the affinity tag, which can then be purified and, after purification, it will be possible to cleave the tag from the protein of interest.

Furthermore, additional genome editing of the heavy chain of immunoglobulin with a red fluorescent protein could also be considered using the method described in this thesis. This would enable the co-expression of double recombinants in the case that the interaction of two different proteins is essential to preserve the biologically activity of the molecule; for example, viral-like particles (VLPs), composed of two or more structural proteins and capable of self-assembly when expressed. Moreover, since mammalian expression systems are highly recommended for the production of VLPs, thanks to their ability to produce more accurate and complex post-translational modifications (Zhu, 2012), our method is preferable, not only for this latter reason, but also for their potential to produce high yields of proteins expressed over time in a stable manner.

The above-described experiments, together with other further studies, will be needed to improve the method and to give rise to new opportunities for its advanced field application. Nevertheless, the pilot experiments contained herein will enable the technique to be developed from its proof of concept to more specific applications.

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Genome editing of a hybridoma cell line via the CRISPR/Cas9 system: A new approach for constitutive high-level expression of heterologous proteins in eukaryotic system

Nicoletta Schibeci Natoli Scialli [a,*], Barbara Colitti [a], Luigi Bertolotti [a], Giulia Pezzoni [b], Eugenio Martignani [a], Maverick Melega [a], Emiliana Brocchi [b], Sergio Rosati [a]

[a] Department of Veterinary Science, University of Turin, Largo Braccini 2, 10095, Grugliasco, TO, Italy
[b] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Bianchi 7, 25124, Brescia, Italy

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ABSTRACT

The power of the CRISPR/Cas9 system has revolutionized genome editing in many fields of biology. These applications have expanded exponentially over recent years, including those regarding protein expression technologies. The CRISPR/Cas9 system avoids random integration of the gene of interest and due to this characteristic can be exploited to obtain a stable cell line for the high-yield expression of recombinant proteins. Here we propose a method to edit a hybridoma cell line for the constitutive expression of proteins of interest using the CRISPR/Cas9 system. First, with the scope of optimizing the method, we replaced part of the light chain of immunoglobulin with the Green Fluorescent Protein (GFP) gene, obtaining a precise knock-in in the hybridoma genome. We confirmed the expression and secretion of GFP into the culture medium via fluorimetric analysis, as well as correct genome editing by RNA sequencing. Then, using the same approach, we included the gene encoding a protein of diagnostic interest, the Bovine Herpesvirus 1 glycoprotein E, in the donor DNA. We obtained a stable clone able to secrete gE protein in fusion with GFP into the culture medium. This result was confirmed by ELISA and Western Blot analysis. This study confirms the suitability of this cell line for the production of proteins of diagnostic interest by stable gene expression in a mammalian system. These experiments will enable the technique to be developed from its proof of concept to more specific applications in the field of infectious disease diagnostics.

1. Introduction

Since their initial development in the 1970s, recombinant DNA technologies for heterologous protein expression have undergone enormous expansion. At present, recombinant protein expression is of paramount importance in a variety of fields, including, but not limited to, basic research, diagnostics, and the pharmaceutical industry (Walsh, 2014,2018; Khan et al., 2016). For example, in the context of the recent COVID-19 pandemic, recombinant DNA technology is playing an indispensable role worldwide in the development of diagnostic kits, prototype vaccines, and pharmaceuticals (Graham, 2020; Zhang et al., 2020).

Prokaryotic expression vectors are the most commonly used systems for the expression of heterologous proteins. They are preferred for a number of reasons including low costs, easy to use techniques, and high protein yields compared with other systems (Demain and Vaishnav, 2009; Zerbs et al., 2009). The major limitation of prokaryotic systems, however, is the lack of any post-translational modification of the expressed protein.

Since post-translational modification is often essential for the correct

Abbreviations: Cas9, CRISPR associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; DMEM, Dulbecco's Modified Eagle Medium; DSB, double stranded break; dsDNA, double-stranded DNA; FACS, fluorescence activated cell sorting; FluD, influenza D virus; BoHV1gE, glycoprotein E of Bovine Herpes Virus tipe 1; GFP, green fluorescent protein; HDR, homology directed repair; HR, homologous recombination; HRP, horseradish peroxidase; mAb, monoclonal antibody; MCS, multiple cloning site; NGS, next generation sequencing; NHEJ, non-homologous end-joining; PAM, protospacer adjacent motif; RNP, ribonucleoprotein-complex; sgRNA, single guide RNA; ssDNA, single stranded DNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease.

* Corresponding author.
E-mail addresses: nicoletta.schibecinatoliscialli@unito.it (N. Schibeci Natoli Scialli), sergio.rosati@unito.it (S. Rosati).

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folding of a protein, an eukaryotic expression system needs to be used when such modifications are required; for example, glycosylation improves protein stability and preserves the biological activity of the protein (Lalonde and Durocher, 2017). The choice of eukaryotic host mainly depends on the characteristics of the protein of interest, since different hosts allow different post-translational modifications (Betenbaugh et al., 2004). In this context a mammalian expression system represents the system of choice (Andréll and Tate, 2013), although yield of production is often its main limitation.

The mammalian expression system can be performed as transient or stable expression. In the context of small-scale protein productions, transient systems are usually preferred, being more simple and rapid. On the down-side, they are more expensive and difficult to standardize, and for this latter reason, they are not recommended when highly reproducible results are required (Wurm, 2004; Bollin et al., 2011). A stable protein expression system requires the integration of the gene of interest and the selection of a cell line capable of expressing the protein over time. However, the genetic modification of mammalian cells in most previously adopted techniques, generally involves the random integration of the gene of interest, sometimes resulting in its insertion into loci with variable levels of transcriptional activity (Büssow, 2015), resulting in very low product yields.

Specific genetic tools, such as the 'clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system', have opened the way to new possibilities in the realm of genome editing. By overcoming the issue of random integration, the CRISPR/Cas9 system allows specific and precise modification of the mammalian cell genome (Jinek et al., 2012; Cong et al., 2013; Black et al., 2017; Parthiban et al., 2019). The key components of the CRISPR/Cas9 system include the Cas9 endonuclease and single guide RNA (sgRNA), which form a ribonucleoprotein (RNP) complex. The sgRNA consists of a 20-nt guide sequence complementary to the target sequence and a scaffold backbone required for RNP complex. The Cas9 protein and the sgRNA can be delivered into target cells through different methods, such as vector-based expression systems, the transfection of RNA, or directly as a Cas9/sgRNA complex. Compared with other methods, the use of RNP complexes, reduce the off-target effects and has been optimized for various cell lines using electroporation and lipid-mediated transfection (Sander and Joung, 2014; Liang et al., 2015). Once delivered into target cells, the RNP complex produces a targeted double-stranded break (DSB) in a specific position of the genome, directly upstream of a requisite 5'-NGG Protospacer Adjacent Motif (PAM). Following a DSB, the most frequent mechanism of DNA repair in mammalian cells is non-homologous end joining (NHEJ), which produces micro insertion-deletion mutations (indels) (Mao et al., 2008; Pannunzio et al., 2018). Alternatively, DNA repair via homologous recombination (HR) may occur, although it is less frequent and requires donor DNA with two flanking regions for HR (Wright et al., 2018). The donor DNA can be in the form of a plasmid, single-stranded DNA (ssDNA) or a double-stranded PCR-derived DNA (dsDNA). A number of studies have confirmed the advantage of ssDNA donors, which increase the integration efficiency by HR (Renaud et al., 2016; Li et al., 2017). The combination of the CRISPR/Cas9 system with HR can allow the insertion of a coding sequence of interest into a precise area of the genome with high transcriptional activity, and preserving similar levels of expression.

The capacity to edit the genome of a hybridoma in a very specific manner leads to the possibility of using such a cell line for the production of a stable mammalian protein expression system, able to secrete the recombinant protein of interest into the medium. Hybridomas are immortalized cell lines derived from the fusion of B lymphocytes with a plasmacytoma cell line (Köhler and Milstein, 1975). They have been developed for the high-level production of monoclonal antibodies (mAb) (Pirofski et al., 1990). Hybridomas are characterized by their ability to express few proteins (the heavy and light chains of immunoglobulin) in large amounts under the control of a strong promoter. Under natural circumstances, the diversity of the antibody repertoire is due to rearrangement events within the immunoglobulin genes VDJC and VJC for the heavy and light chains respectively. The final mRNA also contains a secretory signal peptide that allows for the membrane translocation of the Ig (Dorshkind and Rawlings, 2018).

Hybridomas represent an ideal eukaryotic cell system in which to attempt the CRISPR/Cas9 strategy for protein expression. Recent studies successfully demonstrated the use of the CRISPR/Cas9 system combined with Homology Directed Repair (HDR) to edit the genome of hybridomas (Pogson et al., 2016; Parola et al., 2018, 2019). The aim of such studies is usually the modification of mAbs; for example, to obtain antibodies in a specific format, with the possibility of inserting tags or mutations, or to knock out the antibody constant region in order to express an antibody fragment instead of the whole immunoglobulin (Cheong et al., 2016; Khoshnejad et al., 2018; van der Schoot et al., 2019). To the best of our knowledge, this is the first study reporting the CRISPR/Cas9 genome editing of a hybridoma cell line for the stable expression of heterologous proteins, such as a viral antigen suitable for diagnostic tests development.

The aim of the present study was to edit the genome of a hybridoma cell line using the CRISPR/Cas9 system to produce a stable cell system able to secrete proteins of diagnostic interest into the medium. After the introduction of two DSBs into the hybridoma genome by the CRISPR/Cas9 system, we exploited the DNA damage repair pathway - HDR - to insert the gene of interest using a ssDNA donor.

First, we inserted the gene coding for green fluorescent protein (GFP) to optimize the system for the purpose of this study and as a means to verify correct genome editing. In a second phase, we included a protein of diagnostic interest - the viral glycoprotein E of Bovine Herpes Virus type 1 (BoHV1gE) - in the construct, in order to evaluate the suitability of the system in the field of infectious disease diagnostics.

Bovine herpesvirus 1 (BoHV1) is the causative agent of respiratory disease (Infectious Bovine Rhinotracheitis - IBR), genital disorders (vulvovaginitis or balanoposthitis), transient infertility and abortions in a number of animal species, including cattle, sheep, and goats (Kahrs, 2001). In recent years, the majority of vaccines, used to prevent and reduce the problems associated with BoHV1 infection, are based on DIVA properties. The glycoprotein E (gE) deleted marker vaccines are commonly used in combination with specific gE ELISA tests and protocols, to distinguish between infected and vaccinated animals (Rijsewijk et al., 1999; Mars et al., 2001). The most common approach to recombinant gE production involves the transient plasmid transfection of mammalian cells (Bertolotti et al., 2013). This method is rapid, but generally used for small-scale productions only since protein expression is limited to just a few days, and it is not recommended when highly reproducible results are required (Wurm, 2004; Bollin et al., 2011).

Since recombinant gE expressed in mammalian system has been successfully used in an indirect ELISA for the detection of IgG against the BoHV1gE on pooled milk samples for an IBR surveillance program in a region of north-west Italy (Piedmont) (Bertolotti et al., 2015; Muratore et al., 2017; Colitti et al., 2018), the development of a stable system for the high-yield expression of this protein, would represent a significant advance in the development of tools for IBR disease control. This study was of relevance to develop the technique from its proof of concept to more specific diagnostic applications.

2. Materials and methods

2.1. Hybridomas selection and culture conditions

The hybridoma cell line 3E1 used in this work, developed in a previous study, is known to secrete a monoclonal antibody specific to the hemagglutinin-esterase-fusion protein of influenza D virus (mAb-FluD) (Moreno et al., 2019). The 3E1 cell line derived from the fusion partner NS0 - the murine myeloma cell line originally used for hybridoma production. The NS0 was included in the genetic analysis as reference. Hybridoma cells were cultured in a CO_2 incubator (5% CO_2) at 37 °C, in

high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10 % heat-inactivated fetal bovine serum (Sigma-Aldrich), 2% L-glutamine (Sigma-Aldrich), 1% Antibiotic-Antimycotic solution 100× (Sigma-Aldrich) and 0.1 % Gentamicin solution 50 mg/mL (Sigma-Aldrich). Immediately after cloning, the CRISPR-edited clones were cultured in EmbryoMax MEF Conditioned Media (Sigma-Aldrich) and, following adequate growth, subsequently cultured in complete DMEM. Hybridoma cells were maintained in 7 ml culture medium in T-25 flasks or 15 ml culture medium in T-75 flasks and subcultured every 48 or 72 h, depending on the culture conditions. Hybridoma cells were routinely tested for mycoplasma contamination and resulted negative throughout the study. Specific reactivity of Mab 3E1 was evaluated in indirect ELISA using a semi-purified FluD antigen coated onto the solid phase.

2.2. Hybridoma cells mRNA sequencing

Next Generation Sequencing (NGS) was carried out in order to characterize the VJC gene transcript set of the NS0 and 3E1 cell lines (Moreno et al., 2019). The 3E1 VJC gene set was used to design the donor construct and the sgRNAs. The mRNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions and quantified using a spectrophotometric method using Nanodrop system (Thermo Fisher Scientific). RNA was reverse transcribed into double-stranded cDNA using the Maxima H Minus Double–Stranded cDNA Synthesis Kit (Thermo Fisher Scientific), in accordance with manufacturer instructions. Oligo (dT)18 primers supplied by the kit were used, which selectively anneal to the 3'-end of poly (A)+ RNA, allowing the synthesis of cDNA only from poly(A)+ tailed mRNA. Complementary DNA was quantified using the Qubit 3.0 fluorometer (Thermo Fisher Scientific) using the dsDNA HS Kit (Thermo Fisher Scientific). Samples were used for DNA library preparation using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. cDNA quantification was assessed using the Agilent DNA High Sensitivity chip assay (Agilent Technologies) and the Qubit dsDNA HS Kit (Thermo Fisher Scientific). Paired-end libraries were sequenced using Illumina V2 chemistry and the Illumina MiSeq platform. Sequencing reads were analyzed using Geneious software (ver. 11.1.2) via the resequencing approach. Briefly, reads were aligned to reference belonging to the V, J, and C regions of the mouse immunoglobulin light chain (Mus musculus, chromosome 6 - accession no. NC_000072.6). Once the specific V, J and C regions of the hybridoma were identified, a construct was designed in silico, and used as reference for a second resequencing step. Moreover, the construct was confirmed using a de novo assembly approach. Briefly, reads were assembled into contigs using Velvet software (ver. 1.2.10) (Zerbino and Birney, 2008) with the minimum contig length parameter set to 600 bp, and contigs were aligned to the VJC construct. The same method was carried out to verify the correct insertion of the GFP gene in the GFP-positive clones. The contigs were aligned to the consensus sequences obtained for each clone in order to confirm the VJC or VJ-GFP gene set and the correct coding frame of the final construct.

Optimization of the genome editing protocol, as described thereafter, was evaluated in the first experiments in terms of number of GFP positive cells and established taking into consideration the following parameters: number of transfected cells, µg of ssDNA, minus versus positive strand, transfection method, electroporation parameters.

2.3. Donor construct for HR

We designed a DNA donor construct containing a multiple cloning site (MCS) between the two homology arms, that flank the C gene region of the VJC gene set of hybridoma 3E1 line (HR-MCS), identified after the VJC gene set sequencing of the 3E1 cell line (See supplemental Figure S1). The synthetic DNA fragment HR-MCS was ordered from Eurofins Genomics and cloned into pUC18 to obtain pUC18-HR-MCS (Fig. 1). For the first experiment, the GFP gene was cloned into pUC18-HR-MCS between the two regions for homologous recombination, using BamHI and XhoI (Thermo Fisher Scientific) restriction sites, to obtain pUC18-HR-GFP (Fig. 1). For the second experiment, the BoHV1gE gene subunit encoding the glycoprotein ectodomain was amplified from a synthetic construct developed in previous study (Bertolotti et al., 2013) and cloned into pUC18-HR-GFP between the first homologous recombination region and the GFP gene, using the BamHI (Thermo Fisher Scientific) restriction site, to obtain pUC18-HR-gE-GFP (Fig. 1). All the ligation reactions were performed with T4 DNA ligase (Thermo Fisher Scientific). The correct insertions of all plasmids were assessed by Sanger sequencing.

2.4. Single-stranded DNA production

Since the ssDNA donor is expected to increase HR efficiency, both ssDNAs (plus and minus strands) were obtained using the Guide-it Long ssDNA Strandase Kit (Takara Bio), according to the manufacturer's protocol. Briefly, the dsDNA of interest was amplified via PCR using one regular PCR primer (I-f or I-r) and a second phosphorylated PCR primer (I-f-phos or I-r-phos) (See supplemental Table S1). Only the phosphorylated strand, amplified using the phosphorylated primer, was subsequently degraded by treatment with two different enzymes, Strandase Mix A and Strandase Mix B, at 37 °C for 5 min/Kb. The ssDNA donor

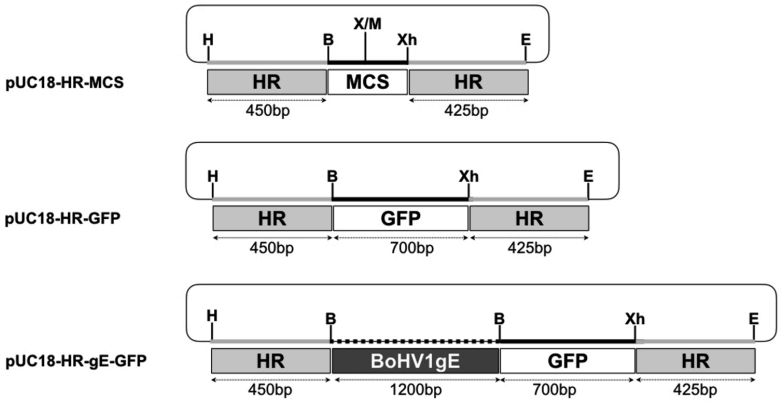

Fig. 1. Schematic representation of the plasmids used in this study. Plasmid pUC18-HR-MCS containing a multiple cloning site (MCS) flanked by two homology arms for homologous recombination (HR). Plasmid pUC18-HR-GFP, containing the green fluorescent protein (GFP) coding sequence, derived from pUC18-HR-MCS. Plasmid pUC18-HR-gE-GFP, containing the Bovine Herpes Virus type 1 glycoprotein E (BoHV1gE) protein coding sequence fused with GFP, derived from pUC18-HR-GFP. Numbers mentioned below the dotted arrows indicate the approximate size of the fragments. Restriction enzymes: B, BamHI; E, EcoRI ; H, HindIII ; M, MluI ; X, XbaI ; Xh, XhoI.

repair construct with the GFP gene flanked by two regions for homologous recombination (HR-GFP) and the ssDNA construct with the BoHV1gE gene fused in frame with the GFP gene (HR-gE-GFP) were obtained using the plasmids pUC18-HR-GFP and pUC18-HR-gE-GFP (Fig. 1), respectively, as DNA templates in the PCR reaction. The quality of each ssDNA was assessed by electrophoresis on a 1.5 % (w/v) agarose-gel. PCR products were column purified using NucleoSpin gel and PCR Clean-Up Kit (Macherey-Nagel), according to the manufacturer's protocol. Finally, each ssDNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) (ssDNA setting).

2.5. Single guide RNA and Cas9/sgRNA complex

The two sgRNA, named sgRNA-tag1 and sgRNA-tag2, were designed in silico according to the guidelines described in the Guide-it Complete sgRNA Screening System Kit (Takara Bio) in order to drive the two DSBs, located up- and downstream of the antibody light constant region of the 3E1 genome (Fig. 2). They were used at the same time in order to obtain two DSBs simultaneously. They were obtained using the Guide-it Complete sgRNA Screening System Kit (Takara Bio), using primers I-sg1 and I-sg2 (See supplemental Table S1), respectively, according to the manufacturer's protocol.

The RNP complexes were obtained by mixing both sgRNA-tag1 and sgRNA-tag2 with the recombinant wild-type *Streptococcus pyogenes* Cas9 nuclease (Takara Bio), according to the protocol provided by the Guide-it Recombinant Cas9 Electroporation Ready Kit (Takara Bio). An in vitro cleavage reaction was set up, according to the Guide-it Complete sgRNA Screening System Kit protocol, in order to verify the specific cleavage of the sgRNAs' target. Briefly, a PCR amplification of the target region was performed, mixed with sgRNA/Cas9 complexes and incubated for 1 h at 37 °C and for 5 min at 80 °C for. The correct cleaved fragments were analyzed by agarose-gel electrophoresis.

2.6. Cell line transfection

The transfection was performed by electroporation using Cell Line Nucleofector Kits C and L (Lonza) with a Nucleofector II device (Amaxa), according to the protocol proposed by the manufacturer. Briefly, 2×10^6 hybridoma cells (line 3E1) were harvested by centrifugation at 90×g at room temperature for 10 min and culture medium removed by aspiration. The pellet was gently resuspended in electroporation buffer, mixed with the Cas9/sgRNA complexes and 1 µg of single-stranded donor DNA (plus strand) (100 µl total volume) and electroporated using the program A-020. Following electroporation, transfected cells were transferred into a 6-well plate containing 2 ml fresh DMEM without antibiotic-antimycotic solution and incubated for 72 h.

2.7. Isolation of the cell line

GFP-positive cells were isolated using the FACSAria IIIu cell sorter (Beckton Dickinson). In the first experiment, single cells were sorted into the wells of multiple 96-well plates containing 200 µl of conditioned medium. After 15 days, the clones were analyzed via inverted fluorescence microscopy and the GFP-positive clones were scaled up into 24-well and then 6-well plates. At this time, fluorimetric analysis of the medium and a NGS sequencing of a subset of GFP positive clones were carried out. The clones were propagated for several passages and analyzed at each passage by flow cytometry to verify the percentage of GFP-positive cells.

In the second experiment, where the ssHR-gE-GFP donor sequence was used, the cells were first sorted to obtain a homogeneous GFP-positive population delivered and grown in a single well of a multiple 6-well plate containing 2 ml conditioned medium. Then, GFP-positive hybridoma clones were singularly cloned by limiting dilution in a 96-well plate and grown in 200 µl conditioned medium. After 15 days, the clones were analyzed using an inverted fluorescence microscope and the GFP positive clones were scaled up into a 24-well plate. At this stage, an ELISA sandwich test and a Western blot using anti-gE specific mAbs

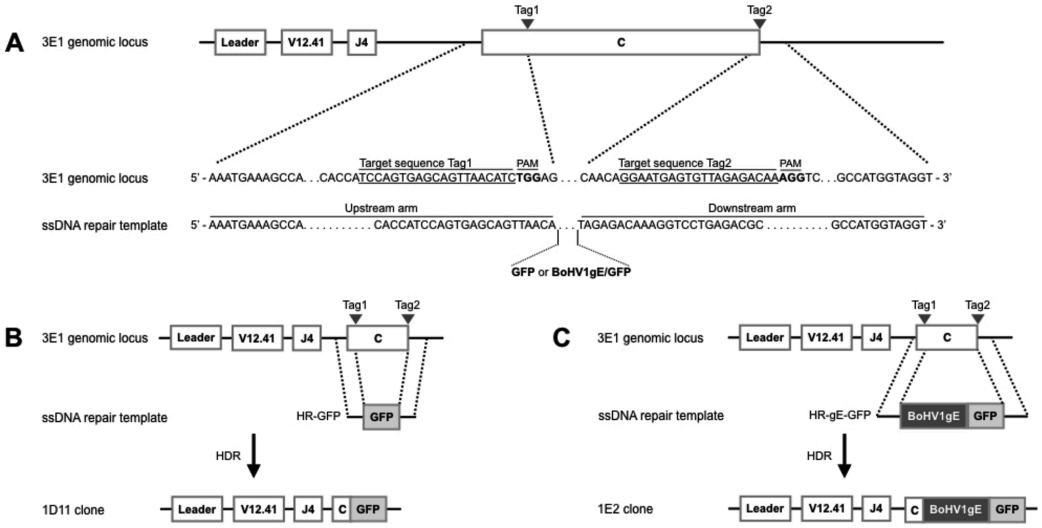

Fig. 2. A schematic representation of HDR after two DSBs in the kappa light chain constant region. The two Cas9/sgRNA complexes (Tag1 and Tag2) resulted in two DSBs upstream and downstream of the light chain C region (black triangle) (the detailed sequence is shown in supplemental Figure S1). (**A**) Single-stranded donor DNAs are used in combination with Cas9/sgRNA complexes for HDR-mediated editing at the target locus. The DSBs were repaired by HDR using: the HR-GFP single-stranded donor (the detailed sequence is shown in supplemental Figure S2) (**B**), or the HR-gE-GFP single-stranded donor (**C**) DNA. BoHV1gE: Bovine Herpes Virus 1 Glycoprotein E gene; C: constant gene segment; GFP: Green Fluorescent Protein gene; J4: joining gene segment; Leader: leader peptide that enables protein secretion, and is subsequently cleaved after translation; V12-41: variable gene segment.

were carried out on replica wells, following standard procedures (Borrè et al., 2004), on both the supernatant and the cell lysate to verify that the expressed protein was successfully secreted. In addition, an indirect ELISA using a set of well characterized bovine sera was carried out to evaluate antigenic properties and the diagnostic potential of the recombinant gE.

2.8. Analysis of clones growth medium

In order to verify the secretion of GFP, the growth medium of each clone was analyzed by fluorescence spectroscopy using the Qubit 3.0 fluorometer (Thermo Fischer Scientific), according to the protocol suggested by the manufacturer. Briefly, 200 µl of growth medium was collected at least 24 h after cell splitting and analyzed using the Qubit fluorometer mode, with the blue excitation light (470 nm) and the reading fluorescence in the green emission channel (510−580 nm). A mock sample was used to assess the background fluorescence of the medium.

In order to quantify the protein secretion, gE concentration was roughly estimated using serial twofold dilution of three clones growth medium coated on solid face and probed with anti-gE specific mAb in indirect ELISA (Borrè et al., 2004). A know concentration of serially diluted recombinant BoHV1gE protein expressed in prokaryotic system and known to be reactive against the same mAb was used as positive control, obtaining a standard curve. A sample of the original 3E1 growth medium was used as negative control.

3. Results

3.1. mRNA sequencing

Sequence analyses were carried out to characterize the light chain gene set in NS0 (fusion partner cell line) and 3E1 cell lines (Moreno et al., 2019). A summary of the results is reported in Table 1. Even if a variable number of reads was obtained in each experiment, the results provided the needed support for the correct identification of the more expressed VJC gene sets.

The 3E1 VJC gene set was used to design the donor construct HR-MCS that contains two homology arms that flank the C gene region of the VJC gene set. The first homology arm includes an intron upstream of the C gene region (Fig. 2-A).

Interestingly, the sequencing data from the NS0 cell line revealed the presence of the V3.12/J2*/C gene set, harboring a stop codon in the J2 region and representing an inactive light chain. The 3E1 cell line showed the co-presence of two light chains. Some reads (36.3 %) assembled a V3.12/J2*/C gene set but the majority of reads (63.7 %) described a functionally active V12.41/J4/C gene set. This latter sequence was identified in the murine chromosome 6.

3.2. GFP expression

Firstly, with the aim of evaluating the efficiency of our method, we used the donor DNA bearing the GFP gene (HR-GFP) (See supplemental Figure S2). A total of 2×10^6 3E1 cells were co-transfected with the Cas9/sgRNA complexes (obtained using both sgRNA-Tag1 and sgRNA-Tag2) and the ssDNA donor repair construct HR-GFP. Seventy-two hours post transfection (See supplemental Figure S3-A), approx. 150 GFP-positive cells were sorted by FACS analysis and singularly transferred into 96-well plates and cultured in conditioned medium (See supplemental Figure S3-B). Following 3 weeks of incubation, we obtained 10 GFP-positive clones. The correct insertion of the GFP gene between the two homologous recombination zones was checked by NGS sequencing in 5 clones, named 1D11, 1D2, 2B2, 2C10 and 2D4. The number of sequencing reads obtained for each clone, as well as for the parental 3E1 hybridoma and the NS0 fusion partner cell line, is reported in Table 1.

Sequence analysis showed the presence of two VJC gene sets for each clone: the functionally active V12.41/J4/GFP and the non-codifying V3.12/J2*/C gene set (also present in the NS0 fusion partner). Notably, the V12.41/J4/C gene set, found in the original 3E1clone, was never recorded.

We analyzed the growth medium of the clones by fluorescence spectroscopy to confirm the secretion of GFP into the medium. Clone 1D11 produced the highest fluorescence value (See supplemental Figure S4).

Considering the results obtained by NGS sequencing and the fluorescence spectroscopy data for the medium of each clone, clone 1D11 was selected for further investigation. In particular, NGS data analysis for clone 1D11 confirmed the substitution of part of the C gene with the GFP counterpart. Moreover, no contigs harboring the GFP gene and mapping to other regions of the murine chromosome 6 were recorded, confirming the absence of off-target insertions of the GFP gene.

Furthermore, clone 1D11 was propagated for several passages to investigate whether the percentage of GFP-positive cells remained constant (See supplemental Figure S3-C). Flow cytometry analysis confirmed that the number of GFP-positive cells was constantly higher than 95 % for up to 8 passages.

An indirect ELISA with a semi-purified FluD antigen coated onto the solid phase was performed to analyze the reactivity of the anti-FluD mAb of the original 3E1 hybridoma. As expected, ELISA reactivity was completely abolished in the 1D11 line, compared with the original line 3E1, confirming the absence of a functional antibody molecule (See supplemental Figure S5).

3.3. BoHV1 glycoprotein E expression

Following the successful gene editing obtained with the line 1D11, we attempted to express a protein of diagnostic interest in fusion with GFP. We transfected 2×10^6 cells of the 3E1 hybridoma cell line using the same Cas9/sgRNA complexes employed in the first experiment and with the ssDNA donor repair construct bearing the BoHV1gE gene fused in frame with the GFP gene (HR-gE-GFP). Seventy-two hours post-transfection, approx. 400 GFP-positive cells were isolated via FACS analysis and cultured in conditioned medium into a single well of a 6-

Table 1
Summary of NGS sequencing results. The number of sequencing reads mapping to the different gene set of light chain immunoglobulin. Average coverage obtained for each clone is reported.

Hybridoma	Reads number	V3.12/J2*/C		V12.41/J4/C		V12.41/J4/C-GFP	
		Mean coverage	(%)	Mean coverage	(%)	Mean coverage	(%)
NS0	2 648 666	9 722 (1872x)	100 %	/	/	/	/
3E1	651 144	340 (67x)	36.3 %	597 (119x)	63.7 %	/	/
1D11	3 882 734	12 497 (3693x)	32.1 %	/	/	26 493 (4922x)	67.9 %
1D2	27 336 352	4 268 (56x)	49.9 %	/	/	4 292 (253x)	50.1 %
2B2	3 753 530	7 468 (2221x)	66.1 %	/	/	3 822 (782x)	33.9 %
2C10	2 608 540	7 156 (1502x)	35.8 %	/	/	12 843 (1.558x)	64.2 %
2D4	2 447 332	10 568 (2.220x)	99.4 %	/	/	65 (8x)	0.60 %

well plate. After one week of incubation (See supplemental Figure S6), 200 vital cells were retrieved and diluted to obtain a single cell/well in 96-well plates. After 3 weeks of incubation, we obtained 11 GFP-positive clones, as detected by inverted fluorescence microscope.

We characterized all 11 clones using immunological methods. First, the culture medium and the lysate from each clone were analyzed by sandwich ELISA, performed with a capture and a horseradish peroxidase (HRP)-labelled anti-gE mAb recognising two different epitopes, and 10 out of 11 clones gave a positive result (See supplemental Figure S7-A). Of the 10 positive clones that gave a positive ELISA result, the one with the highest viability and the fastest cell growth (named 1E2) was further characterized by Western Blot analysis using an HRP-labelled mAb known to recognize a gE linear epitope, confirming the expression of a protein band of about 90 KDa (Fig. 3) (Borrè et al., 2004). In addition, an indirect ELISA was performed using bovine sera (four from naturally infected and three for marker vaccinated animals) and the antigen/antibody complex detected using HRP-labelled anti-bovine IgG (See supplemental Figure S7-B). Finally, a standard curve created in ELISA using serial dilutions of a known positive control showed excellent linearity ($R^2 = 0.999$) and allowed us to quantify the expressed protein (See supplemental Figure S8). The gE concentration in the growth medium of the three different positive clones with the fastest cell growth (including the clone 1E2) was in the range of 70−100 μg/mL

4. Discussion

The CRISPR/Cas9 system is a powerful genome editing tool, with many applications in different fields of biology (Mei et al., 2016; Zhang et al., 2018; Pickar-Oliver and Gersbach, 2019; Wu et al., 2019). The principal advantages of the CRISPR/Cas9 system regard its high editing precision, its low cost, and fast and easy to use technique. Furthermore, its high efficiency and the possibility of obtaining simultaneous multiple DSB with two or more sgRNA are additional advantages not achievable using other tools for genome editing, such as transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) (Gaj et al., 2013).

In the present study, we evaluated the feasibility of using the Cas9 RNP in combination with single strand donor DNA to obtain a stable hybridoma cell line able to express and secrete protein of diagnostic interest into the culture medium.The hybridoma cell line 3E1 was chosen for use in the present study because of the high production yield (more than 1 mg/mL) of the specific mAb when grown in a high-density stationary culture system (CELLine bioreactor flask) (Moreno et al., 2019). The specificity of the CRISPR/Cas9 system allowed us to insert a coding sequence of interest into a precise region of the hybridoma genome, thereby avoiding random integration (Smith et al., 2014). Targeted insertion of a coding sequence into the genome can considerably increase the protein expression level compared with random integration (Lee et al., 2015).

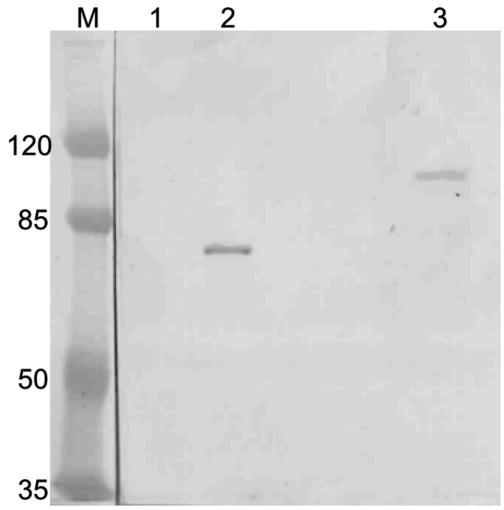

Fig. 3. Western blot analysis using anti gE HRP-labelled mAb. Lane M: prestained molecular weight (sizes are expressed in kDa); lane 1: negative control sample (3E1 clone pellet); lane 2: recombinant BoHV1gE protein; lane 3: 1E2 clone pellet.

In this pilot study, we focused on the kappa light chain constant region, replacing it with a gene of interest and exploiting the original secretion signal, essential for the secretion of recombinant protein into the medium.

We used the RNP complex, due to its notable advantages for genome engineering compared with other CRISPR/Cas9 delivery methods. The RNP complex offers fast editing and it has been demonstrated to be degraded in 24 h, in contrast with the continuous expression of the Cas9 protein from a delivered coding plasmid, which may persist for several days (Kim et al., 2014). The limited activity of the RNP complex reduces off-target mutations and any toxic effects upon the cells.

First, with the aim of evaluating the efficiency of our method, we used a donor DNA bearing the GFP gene (HR-GFP) (Fig. 2-B). Transcriptome sequencing data confirmed, in subset of GFP positive clones, the presence of two VJC gene sets: the functionally inactive V3.12/J2*/C, also present in the NS0 fusion partner cell line used to generate the hybridoma cell line (Moreno et al., 2019); and the functionally active V12.41/J4/GFP. Whereas, the V12.41/J4/C gene set, found in the original 3E1clone, was never recorded, suggesting successful gene editing and the clonal origin of the cell line. Gene expression stability, in terms of the percentage of GFP-positive cells over time, was evaluated in one clone (1D11). Surprisingly, although the percentage of GFP-positive cells was constantly higher than 95 % for up to eight passages, we never obtained 100 % GFP-positive cells by FACS analysis, even after sequential re-sorting purification.

Although this aspect needs to be evaluated over a longer time period, it is probable that the small percentage of GFP-negative cells did not significantly affect the expressed protein concentration. In a recent study, following CRISPR/HDR-hybridoma genome editing, van der Schoot and colleagues did not observe any reduction in antibody production over a period of 1–3 years (van der Schoot et al., 2019).

Following the successful results obtained with the line 1D11, we focused on a different construct to express a protein of diagnostic interest. We confirmed the reliability of this method using a donor DNA sequence bearing the BoHV1gE gene fused in frame with the GFP gene (HR-gE-GFP) (Fig. 2-C). We observed a phototoxic effect in the hybridoma cells following UV light exposure using both the HR-GFP and HR-gE-GFP donors; this photobleaching effect is strongly dependent on the redox environment and it appeared to make those cells more sensitive than other cell lines (Greenbaum et al., 2000; Icha et al., 2017). As a result, in the last experiment, using the HR-gE-GFP donor, it was necessary to minimize the analysis of the transfected cells by fluorescence microscopy to ensure a higher viability rate in the single cell cloning procedure. Single cell sorting, applied in the first experiment was also abandoned in gE-GFP editing and replaced by single cell cloning using a limiting dilution approach which ensured the best cell viability.

In fact, this method damages and stresses cells much less compared with single cell sorting. The 11 GFP positive clones with the highest viability and the fastest cell growth were chosen for further analysis to verify the capability of the clones to express and secrete the protein into the medium.

We confirmed the expression and the secretion of the BoHV1gE protein into the medium in 10 out of 11 clones, using sandwich ELISA (See supplemental Figure S7-A).

Once again, when positive clones were characterized, the specificity of the expressed antigen and the correct secretory pathway was confirmed using highly specific anti-gE mAbs (Borrè et al., 2004). Furthermore, in clone 1E2, we confirmed the expression of a protein band of about 90 KDa by Western Blot analysis (Fig. 3), corresponding to the full expression of the predicted polyprotein V12.41/gE/GFP.

It should be noted that both the capture and detection mAbs were directed against conformational epitopes, known to be of diagnostic relevance. This finding was further confirmed using bovine sera which clearly recognized the recombinant antigen, providing evidence that the actual gene set orientation may confer the correct folding of gE, thereby preserving reactivity of conformational epitopes (See supplemental Figure S7-B).

These pilot experiments will enable the technique to be developed from its proof of concept to more specific applications. In the future, the system could be further improved by genome editing of GFP-expressing cells in order to replace GFP with a protein of interest and thereafter sorting the GFP-negative cells for screening.

In a single attempt, the gene encoding BoHV1gE was also used to replace GFP in 1D11 cells by CRISPR-Cas9 editing, and about 70 GFP-negative cells were single cell sorted. Unfortunately, all clones scored negative for gE expression (data not shown). At present, although the results obtained with the 1D11 line demonstrate stable genome editing, the small, but persistent, percentage of cells negative for GFP expression strongly limited the feasibility of our intention. A level of 5% GFP-negative cells was clearly well above the percentage of CRISPR-Cas9 edited cells achieved in our hands (about 0.075 %, ratio 66:1). We have yet to analyze the transcriptome of GFP-negative clones derived from the 1D11 cell line and, future analysis will be crucial to improve our understanding of the cause of this result.

Further developments will involve replacing the V gene with a protein-based affinity tag by means of a larger gene deletion. Furthermore, additional genome editing of the heavy chain of immunoglobulin with a red fluorescent protein could be considered using similar approach. This would enable the co-expression of double recombinants in the case that interaction of two different proteins is essential to preserve the biologically activity of the molecule, such as a viral-like particle.

5. Conclusions

In conclusion, this study demonstrates the efficient use of the CRISPR/Cas9 RNP complex in combination with single-stranded donor DNA to genome edit a hybridoma cell line. The engineered clones 1D11 and 1E2, expressing GFP and gE/GFP fusion protein, respectively, were able to express and secrete the protein of interest into the culture medium. The low efficiency of HDR was efficiently counteracted through the use of a fluorescent-based sorting procedure, which provided an easy and fast selection method. Further studies are needed to improve the method and give rise to new opportunities for its advanced field application.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2021.110286.

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Chapter 3

A preliminary study on the potential application of the CRISPR/Cas9 system for *Mycoplasma agalactiae* genome editing

Abstract

Until now, very few tools have been developed for the modification of the mycoplasma genome due to the technical difficulty in manipulating the genetic material of this bacterium. Over recent years, the CRISPR-Cas system has emerged as a highly interesting genome editing technology for different fields of biology. Since its discovery, this tool has been applied to edit both eukaryotic and prokaryotic cells, but only recently it was used for the first time to successfully edit the mycoplasma genome. The aim of this study was to investigate in more depth the potential of CRISPR-Cas technology for modifying the genome of mycoplasma.

In particular, we focused on *Mycoplasma agalactiae* (MA), the etiological agent of contagious agalactia in small ruminants. MA constitutes a good candidate for genome editing as a model of ruminant mycoplasmas, as it is known that approx. 18% of its genes have undergone horizontal gene transfer, with the phylogenetically distinct mycoides clusters. Of all MA membrane lipoproteins, the MA P48 protein constitutes one of the specific marker proteins of infection, being the first antigen to be recognized during the early stage of infection. Since the p48 protein has been extensively used in the current (present day) commercially available diagnostic tests, replacement of the P48 gene in the development of a marker vaccine is of particular interest, because it would provide a way to distinguish between vaccinated and infected animals (i.e. DIVA properties). Since it is not known whether p48 is fundamental for MA survival, we identified an orthologous gene, derived from the distantly related *M. canis* (MC), to replace MA p48. Finally, we additionally focused on a second good marker of MA infection: the p40 protein. Since it has been proven that MA can survive without p40, we attempted to replace the p40 gene with an antibiotic resistance marker gene.

This study explored the possibility of using the Cas9 RNP in combination with a donor DNA to mycoplasma genome editing. The final aim was to use this system to knock out and to replace a good marker of infection with the prospective of developing a prototype marker vaccine against contagious agalactia.

3.1 Introduction

3.1.1 Mycoplasma

More than 200 species of mycoplasmas have been classified (phylum *Tenericutes*, class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae*, genus *Mycoplasma*) (Razin & Freundt, 1984; Razin, 1993, 1998; Razin & Hayflick, 2010).

Mycoplasma spp. (from the Greek: *müces* – μύκητας – fungus; *plasma* – πλασμα – formed) are the smallest self-replicating organism. Their minimal genome is of great interest to researchers because it contains the smallest gene-set for sustaining autonomous life. For this reason, mycoplasmas were used as a model when designing the first synthetic bacterial genomes. In 2010, researchers from the J. Craig Venter Institute used these minimal bacteria to create *M. mycoides* JCVI- syn1.0, the first bacterial cell containing a chemically synthesized genome of 1080 kbp capable of self-replicating (Gibson et al., 2010). Thereafter, Hutchison and colleagues identified the minimal set of genes required for autonomous life, and further reduced the JCVI- syn1.0 genome to obtain JCVI-syn3.0, which contains only 473 genes (438 protein-coding genes and 35 RNA-coding genes) in a genome of 531 kbp (Hutchison et al., 2016).

Despite their minimal genome, mycoplasmas are widely distributed in nature as parasites of several species, and they can cause serious diseases in both human and animal populations (Razin et al., 1998). In particular, they are capable of causing persistent and chronic infections, with a predilection for the respiratory and genital tracts, as well as joints, causing respiratory and genital diseases or arthritis, respectively (Razin & Hayflick, 2010; Citti & Blanchard, 2013).

3.1.2 Mycoplasma general features

The specific characteristics of mollicutes are : small cell size (0.2–0.8 μm); low G-C content (around 20–30%); metabolic pathways reduced to minimal activity, compared to other bacteria; 16S ribosomal DNA sequences affiliated with this class; and unique codon usage (Table 4) (Weisburg et al., 1989; Osawa et al., 1992; Razin, 1993, 1998). Furthermore, the most evident feature of mycoplasmas is the absence of a cell wall, meaning that they can be classified neither as gram-positive nor gram-negative bacteria. Indeed, mycoplasmas – and the mollicutes in general – probably derive from gram-positive ancestors (Maniloff et al., 1992; Maniloff, 2002). During their evolution, the

mollicute genome became progressively reduced until it contained just the minimal genes required for survival as commensal or parasite in host cells. Mycoplasmas show a considerable pleomorphism due to their lack of a cell wall. They generally acquire a spherical shape, but many mollicutes can also acquire a pear shape, a flask shape, a filament shape of various lengths or a helical filament shape. The presence of a cytoskeleton-like structure is the most likely reason why mycoplasmas are able to maintain such shapes despite the absence of a rigid cell wall (Razin, 1998). This structure probably participates in cell division, gliding motility and in the localization of adhesins (Razin, 1998). On solid media, mycoplasmas tend to penetrate and grow into the agar (Figure 14-A). As a result, mycoplasmas colonies take on a characteristic "fried egg" appearance (Figure 14-B).

Interestingly, the lack of a cell wall does not simplify the introduction of exogenous DNA into mycoplasma cells. In the 1980s, a low efficiency of chromosomal DNA exchange was demonstrated from direct contact with the mycoplasma cell membrane, as well as from the conjugative transposition of transposon Tn916 from *Streptococcus* (Enterococcus) *faecalis* (Roberts & Kenny, 1987; Barroso & Labarère, 1988). Furthermore, external membrane-associated nuclease activity was found in many mycoplasmas (Minion et al., 1993; Li et al., 2010; Somarajan et al., 2010; Cacciotto et al., 2013). Those nucleases are involved in the acquisition of host nucleic acids required for growth, but they may also contribute to hindering the transformation of mycoplasma with exogenous DNA. However, the transformation of mycoplasma under laboratory conditions can be enhanced using polyethylene glycol (PEG) or electroporation (Razin et al., 1998).

In the last few years, thanks to the technological advancements made in DNA sequencing, horizontal gene transfer (HGT) events have been demonstrated to occur in mollicutes, with important implications with regard to mycoplasma evolution (Citti et al., 2018). Chromosomal transfers were recently described in *Mycoplasma agalactiae* (MA) (Dordet-Frisoni et al., 2013, 2014, 2019). For this reason, MA, as a model ruminant mycoplasma, constitutes a good candidate for genome editing.

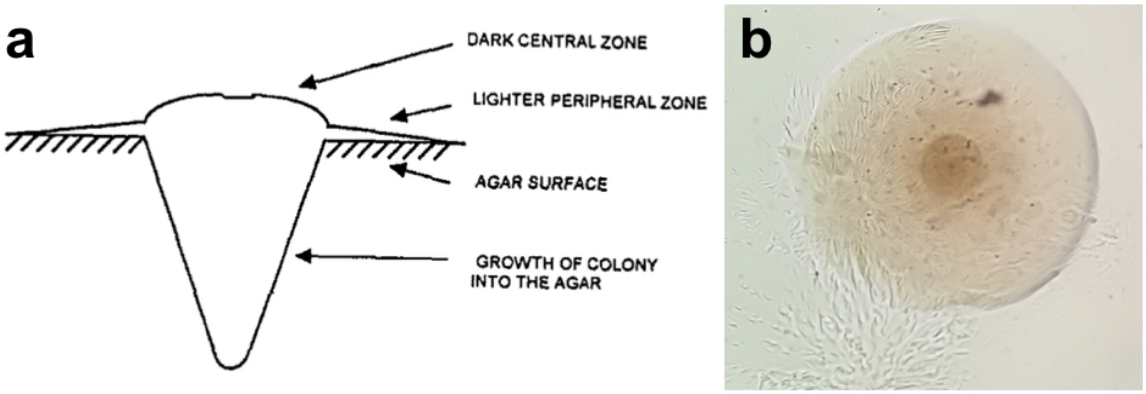

Figure 14: Mycoplasma colony **A**) schematic representation (Miles & Nicholas, 2010); **B**) picture.

Table 4: Mycoplasma general feature compared to other eubacteria. Adapted from Razin et al., 1998.

Property	Mollicutes	Other eubacteria
Cell wall	Absent	Present
Plasma membrane	Cholesterol present in most species	Cholesterol absent
Genome size	580–2.220 kb	1.050– 10.000 kb
G C content of genome	23–40 mol%	25–75 mol%
No. of tRNA genes	30 (*M. capricolum*), 33 (*M. pneumoniae*)	84 (*B. subtilis*), 86 (*E. coli*)
UGA codon usage	Tryptophan codon in *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Mesoplasma*	Stop codon
RNA polymerase	Rifampin resistant	Rifampin sensitive

3.1.3 Mycoplasma genome

The mycoplasma genome consists of a circular, densely packed doubled stranded DNA molecule and its size ranges from 580 kb (*M.genitalium*) to 1380 kb (*M. mycoides*). The variability in its size depends on the presence of repetitive elements or insertion sequence (IS) elements – mobile DNA fragments that may be present in large quantities, and which are generally responsible for the characteristic variability and plasticity of the genome of prokaryotes, even within the same bacteria species (Thomas & Nielsen, 2005).

For decades, the reduction of the mycoplasma genome was considered the only driving force guiding the evolution of mycoplasmas (Woese et al., 1980). HGT had not been considered relevant for the evolution of mycoplasmas, due to their limited occurrence in recombination systems and their low efficiency in the introduction of exogenous DNA (Roberts & Kenny, 1987; Barroso & Labarère, 1988; Rocha et al., 2005). However, the recent discovery of conjugative exchanges of chromosomal fragments between mycoplasma genomes has changed this view (Sirand-Pugnet, 2007; Faucher et al., 2019; Dordet-Frisoni et al., 2019). Interestingly, the integrative conjugative elements (ICEs) present in MA have also been identified in *Mycoplasma bovis* and in other species of the *Mycoplasma mycoides* cluster, suggesting the occurrence of HGT between mycoplasma species belonging to different phylogenetic clusters during co-infection of the same host (Tardy et al., 2015). In particular, it was demonstrated that about 18% of MA genes had been obtained by HGT from species belonging to the phylogenetically distinct *Mycoplasma mycoides* cluster (MA belongs to Hominis, whereas the *Mycoplasma mycoides* cluster belongs to the *Spiroplasma* group; Sirand-Pugnet et al., 2007). Nearly half of these genes (~40%) encode products of unknown function, about 30% encode membrane-associated proteins, and a minority (~15%) have been annotated as pseudogenes. The transferred genes are distributed all over the MA genome, suggesting multiple HGT events and/or intrachromosomal recombination events after their integration. Thus, although the evolution of these organisms has been associated with the overall loss of genes, this important evidence of HGT having taken place shows that these species have maintained the capacity to enrich and evolve their genetic potential specifically through this mechanism.

The total G-C content of the mycoplasma genome is approx. 20-30%. The reduction in G-C content may have been caused by mutation pressure, and in particular this feature may depend on the reduction or lack of Uracil-DNA glycosylase activity (Wieslander et al., 1995) – an enzyme that removes uracil residues from DNA. Consequently, the loss of this pathway may lead, on the one hand, to a decrease in G-C content and, on the other hand, to an increase in the A-T content. The lack of Uracil-DNA glycosylase activity has been demonstrated in most mycoplasma species, including *M. gallisepticum* (31% G-C), *M. capricolum* (25% G-C) and *Ureaplasma urealyticum* (26% G-C)

(Williams & Poilack, 1990). The low G-C content of the mycoplasma genome also influences codon usage in these bacteria. In particular, adenine or thymine bases are preferred in the wobble (3rd) position of the codon. However, this feature is not limited to the third nucleotide position; indeed, A-T rich synonymous codons are generally preferred in mycoplasma spp. (Muto & Osawa, 1987; Ohkubo et al., 1987). Furthermore, mycoplasmas have a unique codon usage. In fact, the UGA codon, which is a universal stop codon in standard genetic code, encodes tryptophan in mycoplasma spp. (Pollack et al., 1997). For this reason, the expression of mycoplasma recombinant proteins, for example, in prokaryotic systems, like *E. coli*, requires the preliminary modification of this codon (Razin et al., 1998).

3.1.4 Mycoplasma genome DNA Repair

The number of genes involved in DNA repair is smaller in mycoplasmas compared with other bacteria. For example, the MA genes involved in HR are as follows: recD, recO and recR are involved in the formation of the presynaptic complex; recA is involved in strand exchange; ruvA and ruvB are responsible for branch migration; and recU and yqgF are involved in Holliday junction resolution. However, among the proteins involved in DNA repair mechanisms, the recA protein, due to its central role, acquires particular significance (Miller & Kokjohn, 1990). Specifically, the function of the recA protein is to catalyze the homologous alignment of two complementary DNA molecules, with consequent strand exchange. The recA protein fills the gap formed by the polymerase system, to form a recA–ssDNA filament. This function is fundamental for homologous recombination; indeed, recombination events are probably recA-dependent in mycoplasmas (Dybvig & Woodard, 1992; Ogasawara et al., 1991; Rocha et al., 2005). The recA gene is almost always present in mycoplasma (King et al., 1994; Fraser et al., 1995; Razin et al., 1998). Different studies have confirmed that homologous recombination occurs in Mycoplasma, although the mechanism has yet to be fully clarified (Dybvig & Woodard, 1992;). Furthermore, it has even been demonstrated that homologous recombination can occur in the absence of complete sets of genes (Rocha et al., 2005). For example, gene disruption by homologous recombination has already been proven in *S. citri* (Duret et al., 1999) and in *M. genitalium* (Dhandayuthapani et al., 1999) – both of which have very few genes involved in DNA repair – whereas the RecA gene is present (Fraser et al., 1995).

Moreover, Allam and colleagues demonstrated that the inclusion of *E. coli* recA in the form of a suicide plasmid favoured HR in the *Mycoplasma mycoïdes* subsp. capri (Allam et al., 2010). This research demonstrated the existence of a number of mechanisms that can be exploited to increase the HR in mycoplasma.

3.1.5 Mycoplasma cell membrane

The mycoplasma cell membrane – the only type of membrane that mollicutes possess – is the sole interface between the cell and its environment, and therefore between the bacterium and its host. Due to the absence of a cell wall, the mycoplasma envelope is less complex compared with that of Gram-negative bacteria (Figure 15). Consequently, mycoplasmas are highly sensitive to lysis by osmotic shock; that said, they are resistant to antibiotics that act on bacteria cell walls, such as fosfomycin, glycopeptides or β-lactam antibiotics (e.g. penicillin).

The major portion of the mycoplasma cell membrane (the hydrophobic core) is composed of lipids, such as phospholipids, glycolipids and cholesterol. However, mycoplasmas are partially, or totally, unable to synthesize fatty acids. As a consequence, their supply depends on the host or on the culture medium, which is generally rich in cholesterol. The mycoplasma cell membrane is richer in lipoproteins compared with other bacteria membranes. Furthermore, it has been shown that a large number of mycoplasma membrane components, including lipoproteins, play a key role in infection, making them good antigens (Chambaud et al., 1999).

3.1.6 Mycoplasma lipoproteins

Due to the absence of a cell wall, mycoplasma lipoproteins anchored in the plasma membrane carry great importance with regards to interactions with the host. Many mycoplasma lipoproteins exert strong immunogenicity due to their surface exposure and due to the presence of an amino-terminal lipoylated structure (Bessler & Jung, 1992). For example, MA lipoproteins, such as P80 (Tola et al., 2001), P30 (Fleury et al., 2001), the variable protein of the *M. agalactiae* (Vpma) family (Glew et al., 2000), and in particular P40 (Fleury et al., 2002) and P48 (Rosati et al., 2000), are often considered to be good markers of infection.

The metabolic pathway of lipoprotein maturation starts when the cysteine residue is modified by diacylglycerol transferase. This enzyme transfers the diacylglyceryl group from glycerophospholipid onto the cysteine sulfhydryl group. Then, the precursor prolipoprotein is cleaved upstream of the modified cysteine by a specific signal peptidase II. As a result, the modified cysteine becomes the first amino acid of the processed protein. Finally, the last step involves N-acylation of the amino terminal diacylglyceryl cysteinyl residue (Sankaran & Wu, 1994). Although the mechanism for lipoprotein translocation to the membrane is unknown, N-acylation probably plays an important role in this mechanism (Buddelmeijer, 2015).

Mycoplasma lipoproteins can undergo two types of variation: I) phase variation, also called ON/OFF switching, which involves variations in protein expression, generally with a site-specific inversion of the promoter upstream of the coding region, which activates or inactivates expression; and II) size variation, involving modifications to protein structure, often by altering the length of the carboxyl-terminal region, or by varying the number of repeated sequences via homologous recombination or slipped-strand mispairing. Both variations can occur spontaneously and frequently (Levinson & Gutman, 1987; Rasmussen et al., 1992; Robertson et al., 1992; Glew et al., 2000; Chopra-Dewasthaly et al., 2008). Size variation plays an important role in mycoplasma pathogenicity; namely, by constantly changing their antigenic mosaic, mycoplasmas are able to evade the host's immune response, thereby enhancing the colonization of host tissues (Dramsi et al., 1993; Rainey et al., 1993).

In the present research, we focused on two well characterized membrane lipoproteins of MA: p40 and p48. The lipoprotein p40 shows a good antibody response, and it plays a key role in cytoadhesion, an important mycoplasma virulence mechanism (Fleury et al., 2002). DNA sequence analysis of p40 has suggested that a high-frequency of phase variations do not occur in this protein since no loci involved in this mechanism have been revealed (Glew et al., 2000). The p48 protein is an immunodominant surface antigen, and it was demonstrated to be an excellent marker of CA infection (Rosati et al., 2000). The p48 protein is highly expressed compared with other MA proteins, and it plays an important immunomodulating role during MA early infection, although its expression is constant throughout all stages of infection (Rosati et al., 1999, 2000). The MA p48 lipoprotein has been demonstrated to exert strong macrophage-stimulatory activity; however, its role in MA has yet to be well defined (Cacciotto et al., 2010; Cacciotto et al., 2013). Finally, the p48 protein is widely used for the preparation of diagnostic kits for MA infection, based on its recombinant form expressed in *E. coli*.

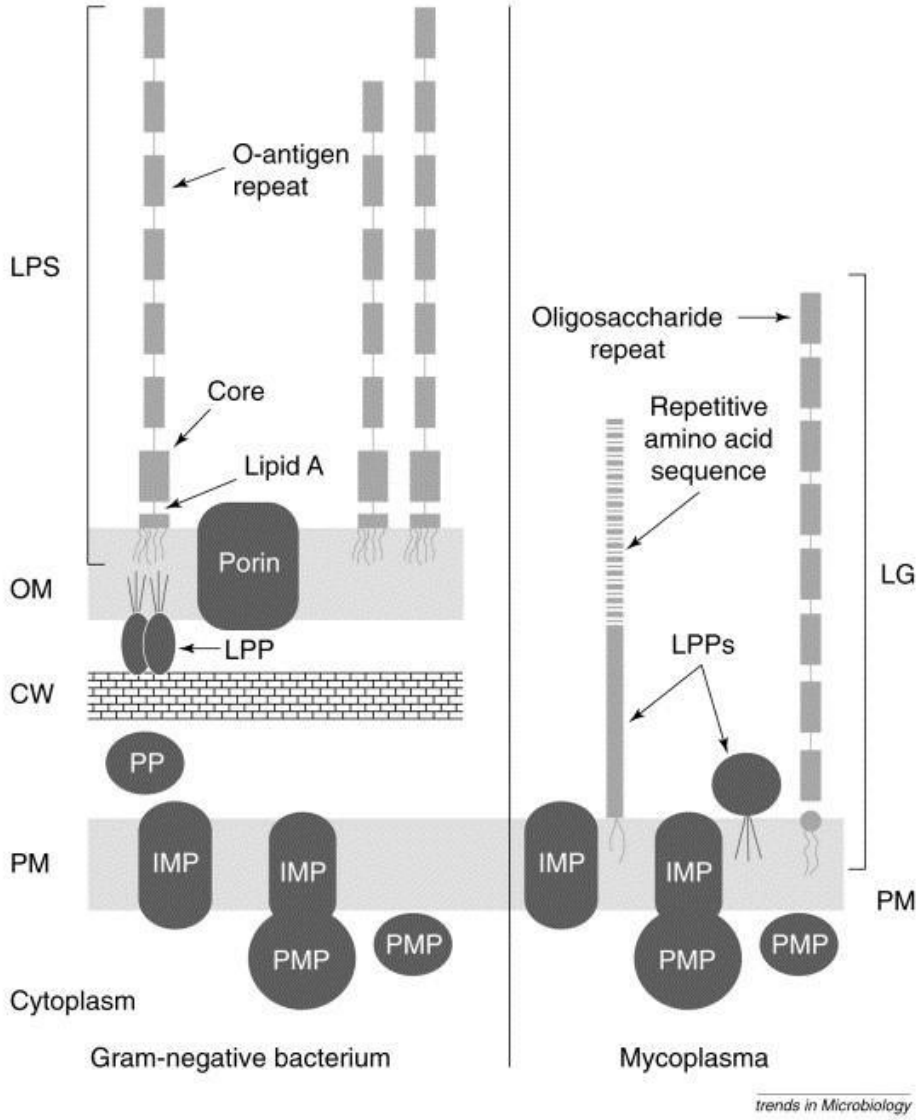

Figure 15: Picture from Chambaud et al., 1999. Differences between the molecular organization of Gram-negative bacteria and mycoplasma envelopes. In contrast with gram-negative bacteria, the lipoproteins in mycoplasmas are bound to the outer surface of the plasma membrane.

Abbreviations: CW, cell wall (peptidoglycan); IMP, integral membrane protein; LG, lipoglycan; LPP, lipoprotein; LPS, lipopolysaccharide; OM, outer membrane; PM, plasma membrane; PMP, peripheral membrane protein; PP, periplasmic protein.

3.1.7 *Mycoplasma agalactiae* and Contagious agalactia

Mycoplasma agalactiae (MA) is the etiological agent of Contagious agalactia (CA) (De la Fe et al., 2005). This pathology causes a chronic disease in sheep and goats widespread across several continents, including North America, Western Asia, North Africa and Europe, being endemic in most Mediterranean countries (Bergonier et al., 1997; Madanat et al, 2001). In Italy, CA is a notifiable disease. Indeed, specific reference is made to CA in national veterinary legislation (art. 97 - Veterinary Police Regulations). However, due to severe restriction measures that must be implemented once a case of CA is reported, it is not always reported by farmers, further aggravating its diffusion and endemicity, as a result of the trade of carrier animals.

CA is characterized by mastitis, polyarthritis and keratoconjunctivitis. The mammary gland is the prime target of MA. The manifestation of symptoms may be acute (unilateral or bilateral mastitis), subacute or chronic, including: fever, inappetence, a swollen udder, transient hypogalactia and/or agalactia. The milk produced is usually greenish–yellow or grayish–blue, and its consistency is first watery, then becomes lumpy. MA also attacks joints, causing polyarthritis, especially in the tarsal and carpal joints. Keratoconjunctivitis, which occurs in approx. 50% of cases, is mostly transient, but in chronic cases it can lead to blindness in one or both eyes. Mortality by CA is not generally high, however, it can result in serious economic loss due to the reduction or complete cessation of milk production and abortions in pregnant animals. The CA incubation period ranges from one week to two months, depending on the virulence of the infectious agent and the resistance of the host. For months, infected animals may spread MA into the environment via infectious milk, urine, faeces, or nasal/ocular secretions. As a result, the disease rapidly spreads through contact between infected and healthy animals. Lambs are usually infected during the lactation period, whereas other animals are infected by the ingestion of contaminated feed or water – in which MA is able to survive. Antibiotic therapy can reduce or totally resolve the symptoms, but in cases of antibiotic resistance the treated animals may continue to spread MA in the environment. At present, the control of CA is difficult due to the general characteristics of mycoplasmas and the absence of any specific marker vaccines – the development of which would greatly aid the practicalities of disease control and the efficacy of a vaccination campaign.

3.1.8 Contagious agalactia vaccines

At present, vaccination against CA is the main tool being implemented to control the disease (Chessa et al., 2009), but no single vaccine has been universally adopted in the farming sector (OIE, 2000). CA vaccines can be divided in two main categories: inactivated vaccines, and live attenuated

vaccines. Live attenuated vaccines are generally more effective, but their use is not permitted in all the countries. The preventive use of live attenuated vaccines reduces the development of clinical signs, but a temporary infection may appear with the agent also being secreted into the milk. By contrast, inactivated vaccines do not have this collateral effect, but the immune response is lower, compared to live attenuated vaccines, and temporary (4-5 months) (Nicolet, 1994; Madanat et al., 2001; Buonavoglia et al., 2008). In Italy, commercial inactivated vaccines are available, and may contain MA only (OVAX-agalactia), or a combination of several mycoplasmas (e.g. AGLOVAX-Intervet, containing: *M. agalactiae*, *M. capricolum* and *M. mycoides*). Moreover, the Italian *Istituti-Zooprofilattici* (Institutes for Veterinary Preventive Medicine) produce autogenous vaccines, obtained using microorganisms isolated from infected animals.

However, each of the abovementioned vaccines has its limits. Indeed, vaccination against CA is known to be inadequate as a result of mycoplasma antigenic variability (Levinson & Gutman, 1987; Rasmussen et al., 1992; Robertson et al., 1992; Glew et al., 2000; Chopra-Dewasthaly et al., 2008). Furthermore, the use of inactivated vaccines or live attenuated vaccines does not permit vaccinated animals to be distinguished from naturally infected animals (Tola et al, 1999; De la Fe et al., 2007). As a result, the eradication of this endemic disease is rendered extremely difficult.

Marker vaccines are also called DIVA vaccines, with DIVA standing for "differentiating infected from vaccinated animals" (van Oirschot, 1999-a). These kinds of vaccines induce a type of immune response that is different from that induced by natural infection. The marker vaccines advantages over classical vaccines are showed in Table 5. Marker vaccines lack at least one antigenic component, thus inducing a distinct immune response compared with that provoked by the wild-type pathogen. Consequently, they permit the precise serological differentiation between infected and vaccinated animals; a feature not achievable through the use of conventional vaccines. The use of marker vaccines, in combination with a serological assay, allows the identification of animals infected with wild-type virus in the vaccinated population. Marker vaccines are generated through the deletion of an entire gene encoding a non-essential immunogenic protein. However, in the case that the protein encoded by the deleted gene is essential for the pathogen's survival, an alternative strategy entails the replacement of the gene with an orthologous counterpart, thereby encoding a functional protein, but which is characterized by a distinct antigenic response.

Until now, the development of marker vaccines against CA has been limited by the lack of efficient genetic tools for editing the mycoplasma genome.

Table 5: Advantages of marker vaccines (adapted from van Oirschot et al., 1996)

- Vaccination does not preclude serological diagnosis on a herd or an individual level
- Seroepidemiology (prevalence and incidence) of infections can be studied in a vaccinated population
- Effectiveness of vaccines can well be evaluated under field conditions
- Combined vaccination-eradication programmes can be implemented
- Marker vaccines may be used without jeopardizing animal trade

3.1.9 The CRISPR/Cas9 system as genetic tool for mycoplasma

Over the last two decades, specific replicating vectors have been developed by a number of research groups as tools for mycoplasmas genetic manipulation (Chopra-Dewasthaly et al., 2005b; Sharma et al., 2015). Since the development of these tools and the publication of evidence demonstrating the feasibility of MA genetic transformation, strong interest in the development of cloning vectors for this pathogen has ensued. However, the poor transformation efficiency, the difficulty in mycoplasma genetic manipulation and the limited number of mycoplasma natural plasmids have all affected the development of tools for the genetic manipulation of mycoplasma, which continue to remain highly limited (Chopra-Dewasthaly et al., 2005b). Considering this context, the CRISPR/Cas9 system has received great interest recently as a potential tool for achieving successful manipulation of the mycoplasma genome (Tsarmpopoulos et al., 2016). Tsarmpopoulos and colleagues used this system to modify the *Mycoplasma mycoides* subsp. capri (Mmc) genome. The scope of this research was to use the yeast genetic tool, such as CRISPR/Cas9, for the modification of the mycoplasma genome. Firstly, they isolated the Mmc genome from the cell, and then they cloned it into *Saccharomyces cerevisiae* yeast. They successfully deleted the mycoplasma glycerol-3-phosphate oxidase-encoding (glpO) gene, using the CRISPR/cas9 system and a DNA template for the HR. Finally, they transplanted the Mmc edited genome into a mycoplasma recipient cell, and confirmed the absence of glpO gene.

However, at present the application of the CRISPR/Cas system to mycoplasma genome editing remains rather limited. The transformation efficiencies, the paucity and the differences in DNA repair pathways used in this class of bacteria are the major obstacles hindering the use of this system. That

said, homologous recombination in MA is known to be feasible since comparative analysis of the genomes of some of the mycoplasma species infecting ruminants revealed that almost 18% of MA genes have been derived by HGT from the so-called, phylogenetically distant, "mycoides" cluster (Sirand-Pugnet et al., 2007). This finding supports the candidacy of MA for genome editing – as a model for ruminant mycoplasmas – such that positive findings and achievements obtained here will be extended to its closest related species.

This research explored the possibility of applying the CRISPR/Cas9 system to mycoplasma genome editing. The final aim was to use this system to knock out the MA p40 and p48 genes with the prospective of developing a prototype marker vaccine against CA.

3.2 Materials and methods

3.2.1 Bacterial culture conditions and total DNA extraction

The *Mycoplasma agalactiae* PG2 (MA-PG2) reference strain (NCBI accession no. NC_009497) (Solsona et et al., 1996) and three Piedmontese field isolates (one *Mycoplasma agalactiae* strain called Stella [MA-S] and two *Mycoplasma canis* strains called M17 and M21), obtained from the Department of Infectious Diseases of the University of Turin, were used in this study. They were grown at 37°C in standard SP4 medium (Tully, 1995) supplemented with 5 mM pyruvic acid (Sigma-Aldrich) and 45 μg/ml cefquinome (Cobactan 4.5%, MSD Animal Health) and, when needed, puromycin (Sigma-Aldrich), gentamicin (Sigma-Aldrich), or tetracycline (Sigma-Aldrich) at the specified concentrations (Table 6). Mycoplasma transformants were selected on SP4 agar plates supplemented with 45 μg/ml cefquinome (Cobactan 4.5%, MSD Animal Health) and specific antibiotics, depending on the ARMG introduced into the mycoplasma. Prior to any subsequent procedure, mycoplasma pellets were obtained by centrifugation at 10.000 x g for 20 minutes at 4°C, and washed three times with PBS 1X. Total mycoplasma genomic DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's protocol.

The plasmids used in this study were amplified using *Escherichia coli* "Top10" competent cells, whereas *E. coli* "One Shot BL21" competent cells were used for protein expression. *E. coli* transformants were grown in standard Luria–Bertani (LB) medium (Sambrook et al., 1989) supplemented with 50 μg/ml ampicillin (Sigma-Aldrich) or other specific antibiotics (Table 6), depending on the ARMG introduced into the bacteria.

Table 6: Standard complements concentration for the SP4 medium preparation.

Complement	**Final concentration**
Gentamicin	50 μg/ml
Puromycin	5 μg/ml
Tetracycline	4 μg/ml
Pyruvic acid	0.005M

Table 7: Primer sequences used in the present study. Restriction sites are underlined and mutated nucleotides (point mutations) are boxed.

Primers used to generate overlapping fragments (*M. agalactiae* p48) to convert TGA in TGG codons	
Name	Sequence (5′ to 3′)
a fw *	TTGGATCCGCATCTTGTGGTGACAAGTACTTT
a′ rv *	TATGAACTGCTTCCCATCCTGATTGGTTAA
b fw *	TTAACCAATCAGGATGGGAAGCAGTTCATA
b′ rv *	GTCAGAAACTGCTGGCCAAGGAATACCACC
c fw *	GGTGGTATTCCTTGGCCAGCAGTTTCTGAC
c′ rv *	TGGGTGTTCTTTATTCCAGTCAATAATACC
d fw *	GGTATTATTGACTGGAATAAAGAACACCCA
d′rv *	TTGAATTCTTTTCTTGTTTCAGAAGCCAACCAGTCAACCAT
Primers used to generate overlapping fragments (*M. canis* p48) to convert TGA in TGG codons	
Name	Sequence (5′ to 3′)
AAA-BamHI-1W-F	TTGGATCCATTTCTTGTGGAGAACCAACAAGTGATTC
AA-1trp-R	GCAATAGTGTTTAATGCTTCCCATGCAGATTGGTTAAATG
BB-2trp-F	CATTTAACCAATCTGCATGGGAAGCATTAAACACTATTGC
BB-2trp-R	GAGCGTAACCTAATTGCCATGAAGCTTGGCTAAC
CC-3trp-F	GTTAGCCAAGCTTCATGGCAATTAGGTTACGCTC
CC-3trp-R	GAATCAGCTAATGCAACCCAACCTGCATCATATC
DD-F	GATATGATGCAGGTTGGGTTGCATTAGCTGATTC

DDD-EcoRI-4W-R		TTGAATTCATTATTTACTATTTTAATTAAATCATCAACAACTG
Primers used to generate the donor DNA HR-P48-canis		
Fragment	Name	Sequence (5′ to 3′)
1°	A-500-f	ATGTTTTCCCTAGTTTATTCTAAG
	aa-new-500-r	CTTGAATCACTTGTTGGTTCTgCACAAGATGCAGCTACCAATGGTACTG
2°	bb-new-p48-f	CAGTACCATTGGTAGCTGCATCTTGTGcAGAACCAACAAGTGATTCAAG
	bb-new-p48-r	CAAAAACCAGGCAGCAAGCaCGGTTTTTTATGTTGATTAAGGATTATTTACTATTTTAATTAAATC
3°	cc-new-500-f	GATTTAATTAAAATAGTAAATAATCCTTAATCAACATAAAAAACCGtGCTTGCTGCCTGGTTTTTG
	C-500-r	CACTTGTAGAATTATTAGGAACCTTTGC
Primers used to generate the donor DNA HR-p48-canis-PAC2		
Fragment	Name	Sequence (5′ to 3′)
1°	HRP48-f	AGCCCGGGGGATCGAAAGGCGGCCGCATGTTTTCCCTAGTTTATTCTAAGC
	HRP48vspac2-R	CGTCTAGATTCAGCAAGCACGGTTTTTTATG
2°	P40pac2vsP48-F	GTGCTTGCTGAATCTAGACGGGGCTAAAG
	P40pac2vsHR2-R	CCGGTTTTTTTTAAGCACCTGGTTTTCTAG
3°	HR2vspac2-F	AGGTGCTTAAAAAAAACCGGGCTTGCTG
	HR2-r	TCCCCCACGAATCTCTAGGCGGCCGCGGTCCAAAAATGAACTTCTGG
Primers used to generate the donor DNA HR-p40-PAC		
Fragment	Name	Sequence (5′ to 3′)
1°	470-BamHI-HRp40-AA-f	TTGGATCCCACAAGCAACTAATGATAAAGAAGGC

	HRp40vspuromyc-AA-r	CTTCTTTAGCCCCGTCTAGATTTCAAGCTTTTTAAGTTCTTC
2°	HRp40vspuromyc-BB-f	GAAGAACTTAAAAAGCTTGaAATCTAGACGGGGCTAAAGAAG
	puromycvsHRp40-BB-r	GAGAAAGCTTTGTCGTTTAAGCACCTGGTTTTCTAGTCATACACC
3°	puromycvsHRp40-CC-f	GGTGTATGACTAGAAAACCAGGTGCTTAAAcGACAAAGCTTTCTC
	470-XhoI-HRp40-CC-r	TTCTCGAGCATCGTCTGTCTCTTCTTCATTC

Primers used to generate sgRNAs	
Name	Sequence (5’ to 3’)
sgRNA-tag1-M	CCTCTAATACGACTCACTATAGGCCATTGGTAGCTGCATCTTGGTTTAAGAGCTATGC
sgRNA-tag2-V	CCTCTAATACGACTCACTATAGGCCTTAATCAACATAAAAAACGTTTAAGAGCTATGC
sgRNA-p40-tag1-m	CCTCTAATACGACTCACTATAGGATGGAAGAACTTAAAAAGCTGTTTAAGAGCTATGC
sgRNA-p40-tag2-v	CCTCTAATACGACTCACTATAGGACTAATTACATATATAGAGAGTTTAAGAGCTATGC

* from Rosati et al., 2000

3.2.2 p48 expression

3.2.2.1 pGex-6H-p48-canis-mut and pGex-6H-p48-aga-mut plasmids

The p48 gene from *Mycoplasma agalactiae* PG2 (MA-PG2) and *Mycoplasma canis* (MC) was amplified by PCR using the 5' and 3' external primers (containing specific restriction sites) listed in Table 7 plus MA-PG2 gDNA or MC gDNA as the DNA template, respectively. We used a BioRad T100™ Thermal Cycler, and the following PCR scheme:

Buffer coral 10x	5.0 µl		95°C 5 min	
mix dNTPs (10 mM each)	1.0 µl		35 cycle	95°C 1 min
Taq HS plus (Qiagen)	0.25 µl			55°C 40 sec
$MgCl_2$	4.0 µl			72°C 90 sec
Primer f 10µM	1.0 µl			
Primer r 10µM	1.0 µl		72°C 10 min	
DNA template (gDNA)	5.0 µl			
H_2O	30.75 µl			

The two PCR products were column purified using the PCR clean-up and Gel Extraction Kit (Macherey-Nagel), according to the manufacturers protocol, and then cloned into the pDrive cloning vector (Qiagen) to obtain PDrive-p48-aga and pDrive-p48-canis. These plasmids were used as templates for site directed mutagenesis, according to the method described by Rosati et al., 2000. Briefly, in order to convert the tryptophan TGA codons (the stop codon for standard genetic code) into TGG codons, point mutations were introduced into the p48 gene. Overlapping fragments were obtained via PCR using specific primers (Table 7), which contained the point mutation. The resulting PCR products were mixed at equimolar concentration and joined together in a second PCR run using the 5' and 3' external primers only (Table 7). All the PCR products were amplified using Taq Hot Start (HS) Plus (Qiagen) in a BioRad T100™ Thermal Cycler.

The quality and the correct size of each DNA fragment was assessed by electrophoresis on a 1.5% (w/v) agarose gel. All the fragments were gel purified before each subsequent step using the PCR Clean-up and Gel Extraction Kit (Macherey-Nagel), according to the manufacturer's protocol. The

final PRC products – p48-MA-mut and p48-MC-mut – were cloned into the pGex-6H expression vector, using BamHI and EcoRI (ThermoFisher Scientific) restriction sites, to obtain pGex-6H-p48-aga-mut and pGex-6H-p48-canis-mut, respectively. All the ligation reactions were performed using T4 DNA ligase (Thermo Fisher). The correct insertion of each plasmid was assessed by Sanger sequencing.

3.2.2.2 *E. coli* transformation

One Shot BL21 Star chemically Competent *E. coli* were transformed via heat shock treatment using pGex-6H-p48-canis-mut and pGex-6H-p48-aga-mut for the expression of MC-p48-mut and MA-PG2-p48-mut, respectively. Both proteins were expressed in fusion with a 26 kDa GST carrier. The presence of a cleavage recognition sequence located upstream of the cloning site permitted the subsequent separation of recombinant protein from GST. Expression in *E. coli* with pGex-6H was isopropyl b-thiogalactopyranoside (IPTG) dependent, and it was performed according to the following protocol: 5 ml of a saturated cell culture of positive clone was grown at 37°C with shaking at 250 rpm in 500 ml standard LB medium supplemented with ampicillin until the OD600 exceeded 0.5 AU. The bacterial culture was then induced by adding 0.5 mM IPTG. After 2 hours of incubation at 37°C with shaking at 250 rpm, the bacterial culture was centrifuged and washed with PBS 1X, and the bacterial pellet lysed using conventional physico-chemical methods. The p48 recombinant protein was recovered in the soluble fraction (S0) and purified by affinity chromatography (Smith & Johnson, 1988). Purity and yield of the recombinants proteins were evaluated by SDS-PAGE and Bradford method (Bradford, 1976), respectively.

3.2.3 Rat Polyclonal Antibody Production

Our proposed experimental procedure for obtaining rat polyclonal antiserum against p48-agalactiae and p48-canis was submitted to the competent authorities, in accordance with DL 26/2014 (implementation of directive 2010/63/EU), and approved.

The polyclonal sera against recombinant p48 of MA and MC were obtained using a 80-day immunization protocol, according to Thermo Fisher Scientific's protocol (thermofisher.com). Briefly, the purified recombinant p48s were emulsified in oily adjuvant or Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA), and injected subcutaneously at a dose of 0.1mg antigen per rat. Two Sprague-Dawley rats were immunized against each antigen. Before the first immunization,

the pre-immune serum of each animal was collected and used as a negative control for subsequent analyses. After 20 days, the same antigen dose was re-injected. Five days after the first booster, serum samples were obtained and analyzed by ELISA, according to the protocol described in a previous research (Rosati et al., 2000). After the second booster, another ELISA test was performed to verify the reactivity and to quantify the immune sera, using the pre-immune serum as negative control. Animals were individually housed in separate cages. The complete 80-day immunization protocol is schematized in the Table 8.

Table 8: The 80-day immunization protocol.

Procedure	**Protocol day**	**Description**
Control serum collection	Day 0	Pre-immune bleed (~1 mL per rat)
Primary injection	Day 1	Immunize with 0.10 mg antigen in CFA, SQ 4 sites
1st booster	Day 21	Boost with 50µg antigen in IFA, SQ 4 sites
Test bleed	Day 26	Test bleed (~1 mL per rat)
2nd booster	Day 42	Boost with 50µg antigen in IFA, SQ 4 sites
Test bleed	Day 48	Test bleed (~1 mL per rat)
ELISA	Day 60	ELISA titration of pre-immune and test bleed;
Decision	Day 64	Verify disposition of rats; decide whether to continue or terminate.
Expiration date	Day 80	Last bleed (2 mL per rat). The sample is tested on the same day. If blood quantity is insufficient, the euthanasia of the animals will be evaluated such that a larger quantity of blood and an adequate quantity of antibodies may be obtained.

3.2.4 Donor DNA for HR in Mycoplasma

The concatenation method for obtaining the DNA template is schematized in Figure 16. Briefly, in a first round of PCR, overlap sequences were incorporated up- and downstream of the amplicon using specific primers. The overlapping amplicons were mixed at equimolar concentration, and then joined together, using the 5' and 3' external primers only, in a second round of PCR. The fragments, with their overlapping ends, act as primers for each other. All the PCR products were amplified using Taq Hot Start (HS) plus (Qiagen) in a BioRad T100™ Thermal Cycler.

The quality and the correct size of each DNA fragment was assessed by electrophoresis on a 1.5% (w/v) agarose gel. All the fragments were gel purified before each subsequent step. PCR products smaller than 2kb were gel purified using the Nucleospin gel and PCR Clean-up kit (Makery-Nagel), according to the manufacturer's protocol. Fragments bigger than 2kb were gel purified using the TOPO® XL PCR Cloning Kit (Thermo Fisher Scientific), which involves crystal violet staining, according to the manufacturer's protocol. Briefly, a 0.8% agarose gel was prepared adding 30 µl of 2 mg/mL Crystal Violet solution. Then, 8µL 6X Crystal Violet Loading Buffer was added to 40µl of the PCR amplification and loaded onto gel. The gel was run at 80 volts until the crystal violet in the gel had run about a quarter of the way UP the gel (crystal violet migrates towards the negative pole). The thin blue PCR product is faintly visible under normal light and it moves down the gel. Finally, the band was carefully excised from the gel using a razor blade and purified according to standard procedures. Following purification, the final concatenated fragment was cloned into a specific plasmid. All the ligation reactions were performed using T4 DNA ligase (Thermo Fisher). The correct insertion of all plasmids was assessed by Sanger sequencing.

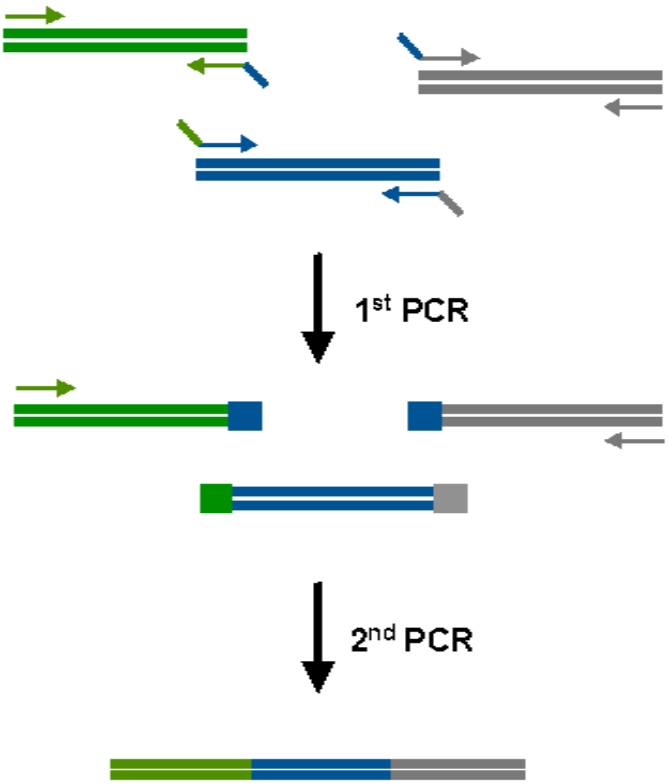

Figure 16: In a first round of PCR, overlapping sequences were incorporated up- and downstream of the amplicons using specific primers. Then, DNA fragments contain homologous overlapping ends are assembled in a second run of PCR, using the 5' and 3' external primers only.

3.2.4.1 Plasmid TopoTA-HR-p48-canis

Three overlapping fragments were obtained. The first and the third fragments, containing the regions upstream (~500 bp) and downstream (~500 bp) of the MA-PG2 p48, respectively, were obtained using the MA-PG2 gDNA as DNA template and the primers listed in Table 7 The second fragment, containing the MC p48 gene (~1270 bp), was obtained using the MC gDNA as DNA template and the primers listed in Table 7. These three fragments were joined together in a PCR run, using the 5' and 3' external primers A-500-f and C-500-r (Table 7) only, to obtain the fragment called HR-p48-canis (~2270 bp). HR-p48-canis was then cloned into TopoTA vector (Thermo Fisher) to obtain TopoTA-HR-p48-canis (Figure 17).

3.2.4.2 Plasmid TopoTA-HR-p48-canis-PAC2

Three overlapping fragments were obtained. The first fragment containing the region upstream of the MA-PG2 p48 in fusion with the MC p48 gene (~1770 bp) was obtained using TopoTA-HR-p48-canis as DNA template and the primers listed in Table 7. The second fragment, containing the puromycin N-acetyltransferase (PAC) gene under the control of the MA p40 promoter (~790 bp), was obtained using the plasmid pMiniO-pur as DNA template and the primers listed in Table 7. The third fragment, containing the region downstream of the p48 of MA-PG2 (~500 bp), was obtained using MA-PG2 gDNA as DNA template and the primers listed in Table 7. These three fragments were then joined in a PCR run, using the external primers HRP48-f and HR2-r (Table 7) only, to obtain the fragment HR-p48-canis-PAC2 (~3050 bp). Finally, this fragment was cloned into TopoTA vector (Thermo Fisher) to obtain TopoTA-HR-p48-canis-PAC2 (Figure 17).

3.2.4.3 Plasmid TopoTA-HR-p40-PAC

Three overlapping fragments were obtained. The first and the third fragments, containing the regions upstream (~470 bp) and downstream (~470 bp) of the p40 of MA-PG2, were obtained using the MA-PG2 gDNA as DNA template and the primers listed in Table 7. The second fragment, containing the puromycin N-acetyltransferase (PAC) gene under the control of the p40 promoter of MA (~790 bp), was obtained using the plasmid pMiniO-pur as DNA template and the primers listed in Table 7. Then, these fragments were joined in a PCR run, using the 5' and 3' external primers only, to obtain the fragment called HR-p40-PAC (~1730 bp). The fragment HR-p40-PAC was cloned into TopoTA vector (Thermo Fisher) to obtain the plasmid TopoTA-HR-p40-PAC (Figure 17).

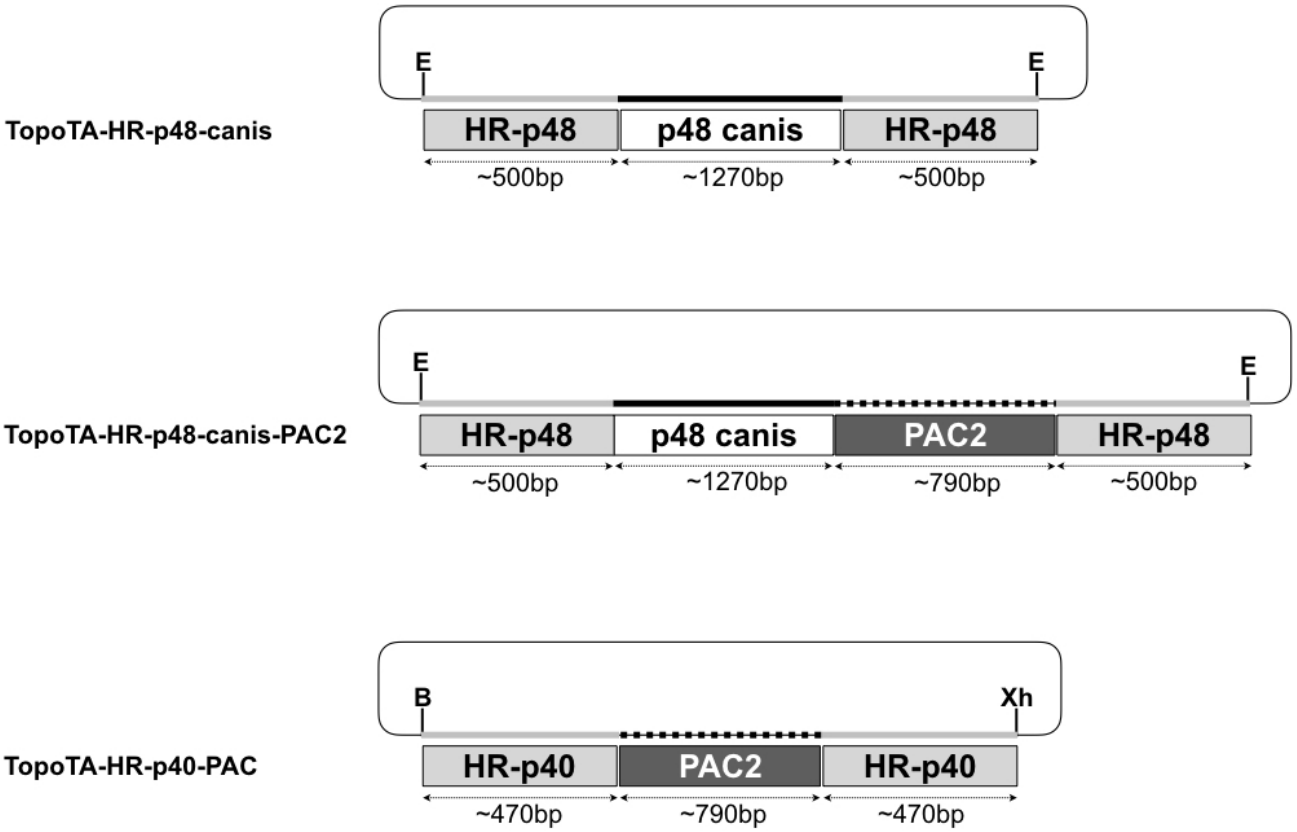

Figure 17: Schematic representation of the plasmids used in this study. Plasmid TopoTA-HR-p48-canis containing the MC p48 gene flanked by two homology arms for homologous recombination (HR) upstream and downstream of the MA-PG2 p48. Plasmid TopoTA-HR-p48-canis-PAC2, containing the MC p48 gene and the puromycin N-acetyltransferase (PAC) gene under the control of the MA p40 promoter. Plasmid TopoTA-HR-p40-PAC, containing the PAC gene under the control of the p40 promoter of MA flanked by two homology arms for homologous recombination (HR) upstream and downstream of the MA-PG2 p40. Numbers mentioned below the dotted arrows indicate the approximate size of the fragments. Restriction enzymes: B, BamHI; E, EcoRI ; Xh, XhoI.

3.2.5 ssDNA donor construct

The ssDNAs were obtained using the Guide-it Long ssDNA Strandase kit (Takara), according to the manufacturer's protocol, as described in the previous section (2.2.4). Briefly, the dsDNA of interest was amplified via PCR using one regular PCR primer and a second phosphorylated PCR

primer, using specific plasmids as DNA templates (Figure 17). Each PCR reaction was analyzed on a 1.5% agarose gel, then column purified using the PCR Clean-up Gel Extraction Kit (Macherey-Nagel), according to the manufacturer's protocol. All PCR products were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) (dsDNA setting). Each PCR product was independently subjected to two short and consecutive Strandase reactions to obtain ssDNA. Finally, 5 µl of each ssDNA sample was analyzed on a 1.5% agarose gel, including 100 ng of the dsDNA substrate in a separate lane as control. All products were purified and subsequently quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) (ssDNA setting).

3.2.6 Single Guide RNA

The two sgRNAs, named sgRNA-tag1-M and sgRNA-tag2-V, were designed in silico according to the guidelines described in the Guide-it Complete sgRNA Screening System Kit (Takara Bio) in order to drive the two DSBs, located up- and downstream of the p48 gene of the MA genome. While, the two sgRNA, named sgRNA-p40-tag1-m and sgRNA-p40-tag2-v, were designed in order to drive the two DSBs, located up- and downstream of the p40 gene of the MA genome.

The sgRNAs were obtained using the Guide-it Complete sgRNA Screening System Kit (Takara Bio), according to the manufacturer's protocol, as described in the previous section (2.2.5), using primers listed in Table 7. The RNP complex was obtained using the recombinant wild-type *Streptococcus pyogenes* Cas9 nuclease (Takara Bio) and both sgRNA-*1 and sgRNA-*2, according to the protocol provided by the Guide-it Recombinant Cas9 Electroporation Ready Kit (Takara Bio), as described in the previous section (2.2.6).

3.2.7 *Mycoplasma agalactiae* transformation

3.2.7.1 Transformation via Electroporation

Transformation was performed by electroporation, using mycoplasma electroporation buffer (EB; 272 mM sucrose, 8 mM HEPES, pH 7.4) and a Nucleofector™ II device (Amaxa), according to the protocol described in Chopra-Dewasthaly et al., 2005b. Briefly, mycoplasma cells from 1 ml of late log-phase culture (~10^8/10^9 CFU/ml) were harvested by centrifugation at 10.000 x g for 20 minutes

at 4°C and washed three times with chilled EB, before final resuspension in 100 µl of chilled EB. At this stage, specific plasmid (as positive control) or the Cas9/sgRNA complex together with 1 µg of donor DNA were added to EB. Then, the cells were transferred in pre-chilled electro-cuvettes and incubated on ice for a maximum of 30 minutes. Finally, they were electroporated using bacterial programs. Immediately after the electric pulse, 900 µl of chilled SP4 broth were added directly into the electro-cuvette. The electro-cuvettes were kept on ice for max 15 minutes, then at 37°C for 2 hours. After 2 hours of incubation, 300 µl of cell suspension were spread onto SP4 agar plates, and incubated at 37°C. The plates were examined for up to a maximum of 9 days using a stereomicroscope.

3.2.7.2 Transformation via Polyethylene Glycol-8000

Mycoplasma agalactiae were transformed according to the protocol developed in the laboratories at INRA/ENVT - Interaction Hôtes-Agents Pathogènes (Toulouse, France).

10 µl of a mycoplasma stock culture were inoculated into 1ml SP4 medium and incubated at 37°C for 24 hours. Then, 10 µl of this pre-culture were inoculated into 1ml SP4 medium and incubated at 37°C for 24 hours. Mycoplasma cells were centrifuged at 10.000 x g for 20 minutes at 4°C. The pellet was washed three times with 1ml cold PBS, then centrifuged at 10.000 x g for 10 minutes at 4°C. The pellet was resuspended in 375 µl cold $CaCl_2$ 0.1 M, and the suspension was incubated on ice for 30 minutes. Then, 1 ml of 50% PEG-8000 was mixed with the Cas9/sgRNA complex, 1 µg single strand donor DNA and 10 µg yeast tRNA (Thermo Fisher). Then, 100 µl mycoplasma cells suspension were added to PEG-mix, homogenized gently and incubated for precisely 1 minute at room temperature. Then, 5 ml of the appropriate mycoplasma medium were added and mixed gently. The mycoplasma suspension was incubated at 37° for 3 hours. After this time, the pellet was harvested by centrifugation at 8000 x g for 8 minutes at room temperature, then resuspended in 1 ml of Mycoplasma medium. 300 µl of transformation medium was spread onto SP4 agar plates, and incubated at 37°C. The plates were for examined up to a maximum of 9 days using a stereomicroscope.

3.2.8 Dot blot of mycoplasma colonies

The mycoplasma suspension culture was plated on SP4 agar medium (petri dishes), using a specific dilution to obtain well-isolated colonies. After 4 or 6 days of growth, depending on the colonies' dimensions, colonies were blotted onto a nitrocellulose membrane (SigmaAldrich) by applying the membranes directly onto the petri dishes for 3 minutes. The membrane was stained using Ponceau-S solution (Sigma Aldrich) to verify the effective blotting, then de-stained by rinsing with large quantities of demineralized water. The membrane was blocked with 5% milk powder diluted in TBS 1X for 2h at room temperature under constant gentle agitation. The membrane was washed three times with TBS 1X. It was then incubated with anti-p48 primary antibodies diluted in blocking solution for 2h at room temperature under constant gentle agitation. After washing three times with TBS 1X, the membrane was incubated with anti-rat secondary antibodies (Thermo Fisher) diluted in blocking solution for 2h at room temperature under constant gentle agitation. After a further three washes, the membrane was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) or 4-chloro-1-naphthol solution.

3.2.9 Mycoplasma clone mRNA sequencing

Next Generation Sequencing (NGS) was carried out in order to analyze the genome of the mycoplasma colony. The total mycoplasma DNA was extracted as described previously in section 3.2.1 (Bacterial culture conditions and total DNA extraction). The sample was used for DNA library preparation using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. After DNA purification and quantification, paired-end libraries were sequenced using Illumina V2 chemistry and the Illumina MiSeq platform. Sequencing reads were analyzed using Geneious software (ver. 11.1.2) via the resequencing approach. Briefly, reads were aligned to reference strains (*M. agalactiae* PG2 - accession no. NC_009497.1), and a construct was designed in silico and confirmed using a de novo assembly approach. Sequences were assembled into contigs using Velvet software (ver. 1.2.10) (Zerbino & Birney, 2008).

3.3 Discussion

The principle of marker vaccination is to provide a means of differentiating between the specific antibodies produced by vaccinated animals vs infected animals. This is made possible because the immunizing agent of a marker vaccine lacks a specific marker antigen, and as a consequence induces a distinct immune response to that of the wild-type pathogen (van Oirschot et al., 1986, 1999-a). Consequently, the genetic modification of the pathogen by deletion of an antigenic protein constitutes the first step in the long process needed for the development of a prototype marker vaccine.

At present, the extent to which the genome of mycoplasmas can be edited using the CRISPR/Cas9 system is rather limited (Tsarmpopoulos et al., 2016). This is caused by the various difficulties related to the CRISPR/Cas9 system: specific mycoplasmas codon usage is not compatible with the use of classical CRISPR expression vectors, so specific plasmids need to be optimized before their use in mycoplasmas (Razin, 1993, 1998); the Cas9-induced double-stranded break may be fatal to many bacteria, including mycoplasmas, due to the absence or reduction of the non-homologous end joining (NHEJ) pathway for DNA repair (Zhao et al., 2020). Regarding this last problem, the use of a coding plasmid expressing Cas9 also increases its toxicity because it can persist within the cell for several days (Kim et al., 2014). By contrast, the sgRNA/Cas9 RNP complex has been demonstrated to degrade in 24 hours; thus, reducing off-target mutations and the toxic effect upon cells.

The aim of this research project is to explore the use of the CRISPR system to modify the MA genome with the final goal of obtaining new tools for the control of diseases caused by this microorganism, and in particular a prototype marker vaccine.

Very few genetic tools are currently available for the manipulation of MA and mycoplasmas in general (Halbedel & Stülke 2007). For this reason, the CRISPR system, given all its advantages (low costs, easy to use, simple to adapt to new targets), has been receiving increasing attention from researchers interested in editing the mycoplasma genome. Indeed, should this system be successfully applied to the context of mycoplasmas, it would provide researchers with an indispensable tool for investigating this fascinating microorganism – one of the smallest self-replicating cells, and which was used as a model for the design of the first synthetic bacterial genomes (Gibson et al., 2010).

Despite their minimal genome, mycoplasmas are widely distributed in nature as parasites of several species, and they can cause serious diseases in both human and animal populations (Razin et al., 1998). As a consequence, the development of new tools (e.g. DIVA vaccines) for the control of animal or human diseases caused by mycoplasmas is of great importance, and the CRISPR system

would appear to constitute a promising route for efficiently editing the mycoplasmas genome for the accomplishment of this aim.

At present, one noteworthy method used for the discovery, genetic modification and functional characterization of microorganisms for which genetic editing tools are not yet available, or are limited, such as mycoplasma, involves cloning the entire genome into yeast *Saccharomyces cerevisiae* (Kouprina & Larionov, 2003; Benders et al., 2010; Karas et al., 2012). In this way, the cloned genome can be modified using the wide range of genetic tools available for yeasts, such as Tandem Repeats coupled with Endonuclease Cleavage (TREC) (Noskov et al., 2010), TREC-IN (Chandran et. al., 2014), and CRISPR/Cas9 (DiCarlo et al., 2013; Tsarmpopoulos et al., 2016). However, of the above-mentioned systems, use of the CRISPR system to edit the genome cloned into yeast would be preferred, despite it being less efficient, because it is significantly faster and more precise (Noskov et al., 2010; Tsarmpopoulos et al., 2016; Ruiz et al., 2019). The TREC method, for instance, involves a number of different steps: the first is the integration of a URA3 marker gene at the target locus via HR; the second is the expression of I-SceI (a rare cutter endonuclease) to obtain a DSB close to the URA3 marker that enhances a second HR event to generate the deletion. Thus, in comparison with this very time-consuming method, the CRISPR system is much faster and simpler.

However, a final important step is necessary to take full advantage of genome modification within yeast cells: the modified genome must be re-transplanted back into the original cell or into a recipient cell. Interestingly, Tsarmpopoulos and colleagues were successful in re-transplanting the *Mycoplasma mycoides* subsp. capri (*Mmc*) modified genome into a mycoplasma recipient cell (*Mycoplasma capricolum*) and confirmed correct genome editing (Tsarmpopoulos et al., 2016). However, although the transplantation method into yeast has been optimized and tested many times, a key problem remains, that is the instability of the transplanted genome, since large degradation events are likely following several rounds of yeast propagation (Benders et al., 2010; Noskov et al., 2010; Rideau et al 2017). Moreover, genome re-transplantation from yeast into bacterial recipient cells is very laborious, difficult to obtain, and not very efficient (Lartigue et al., 2009; Tsarmpopoulos et al., 2016). For these reasons, although the strategy of cloning genomes of interest into yeast cells – for which more editing tools are available – may be considered innovative and useful for mycoplasma genome editing, it is very time consuming and expensive, and as such is currently unpractical by most academic laboratories. It should be added that, during my research project attempts to use this strategy on *M. agalactiae* was carried out by C. Citti et al. (INRA/ENVT, Toulouse, France), without success (C. Citti personal communication; unpublished)

Other strategies exploiting the CRISPR system for genome editing may involve the use of specific vector-based expression systems or a preformed RNP complex.

Here, considering the finding that approx. 18% of the genes in MA originated from horizontal gene transfer from phylogenetically distinct mycoides clusters, which probably took place during co-infection of the same host (Tardy et al., 2015), we deemed MA to be a good candidate for genome editing as a model of ruminant mycoplasmas. Thus, this thesis focuses on the application of the RNP-based CRISPR/Cas tool to MA.

In this study, we tried different strategies for mycoplasma transformation using the Cas9 RNP in combination with double and single strand donor DNA, unfortunately without any positive results.

The initial strategy was to attempt replacement of the MA p48 lipoprotein with an orthologous protein that allows protein function to be preserved in modified bacteria, but produces a distinct antigenic response. Initially, the use of antibiotic resistance (AR) as a selection marker was avoided to increase the security of a future prototype vaccine as the use of AR can limit the possibility of obtaining authorization for vaccine experimentation.

Two *Mycoplasma agalactiae* strains (MA-PG2 and MA-S) were chosen for transformation using the CRISPR RNP complex and donor DNA containing the orthologous gene.

First, sequence analyses were carried out to analyze the MA-PG2 and MA-S genome regions flanking the p48 gene. The regions for HR matched in both strains. Consequently, both strains could be transformed using the same DNA template containing the two regions flanking the p48 gene. Subsequently, p48 amino acid sequences from different mycoplasma non-pathogens or with different host-pathogenicities (obtained from the GeneBank database) were analyzed and compared against the MA p48 sequence with the aim of identifying the best orthologous protein. Of all the sequences analyzed, MC p48 seemed to be the best candidate as an orthologous protein. Indeed, its amino acid sequence is very different compared with that of MA p48, thus, this high degree of difference might result in a distinct antigenic response. Furthermore, MC p48 contains two conserved motifs also present in MA p48 sequence – SFNQS and IGVD-DQ – used for the individuation of a family of bacterial lipoproteins and putative products (Table 9) (Rosati et al., 1999).

In a previous research, the Department of Infection Diseases of Veterinary Medicine of Turin University isolated two *Mycoplasma canis* strains: MC-17 and MC-21. The genome sequence of the *M. canis* reference strain LV, retrieved from the database (accession no. NZ_CP011368.1) was used to design two primers for the PCR amplification of both MC-17 and MC-21 p48 genes, which were used for this study (AAA-BamHI-1W-F and DDD-EcoRI-4W-R, sequence in table 7). The PCR products were subsequently sequenced and analyzed. The two p48 amplified sequences of both MC-17 and MC-21 were found to match each other perfectly. The presence of the two conserved motifs and the high level of amino acid diversity as compared with the MA p48 sequence were also confirmed (Figure 18).

Table 9: Bacterial lipoproteins bearing SLA[a] motifs (adapted from Rosati et al., 1999).

Species and product	Function(s)	SFNQS motif (position)	IGVD-DQ motif (position)
M. agalactiae P48	Surface antigen	DESFNQS (78–84)	FIIGVDADQ (318–326)
M. fermentans M161Ag or MALP-404	Inducing cytokine production by human monocytes	DKSFNQS (75–81)	YVIGVDSDQ (290–298)
M. hyorhinis P47 (formerly *M. arginini* Ag243-5)	Metastasis-promoting activity	DKSFNQS (72–78)	YLIGVDTDQ (306–314)
M. genitalium MG040	Putative membrane lipoprotein	DKSFSEM (54–60)	AIIGVDSAQ (358–366)
M. pneumoniae MG040 homolog	Putative membrane lipoprotein	DKSFSQM (54–60)	AVIGVDSAQ (359–367)
Treponema pallidum TMPC	Membrane lipoprotein C, 35-kDa antigen	DKSFNQQ (53–59)	WVIGVDRDQ (253–261)
Bacillus subtilis YUFN	Putative membrane lipoprotein	DKSFNQS (43–49)	WVIGVDKDQ (246–254)
Borrelia burgdorferi [b] BMPA	Basic membrane protein A, immunodominant antigen	DKSFNES (41–47)	YIIGVDEDQ (237–245)
B. burgdorferi [b] BMPB	Basic membrane protein B, immunodominant antigen	DKSFNSS (40–46)	YVIGADQDQ (242–250)
L. monocytogenes TCSA	CD4 TCSA	DRSFNQS (54–60)	_[c]

[a] , SLA, selective lipoprotein-associated motifs.

[b] , BMPA and BMPB of *Borrelia garinii* and *Borrelia afzelii* were omitted even if they bore both SLA motifs.

_[c], the TCSA (T-cell-stimulating antigen published sequence) is truncated immediately upstream of the site that might contain the SLA-2 motif

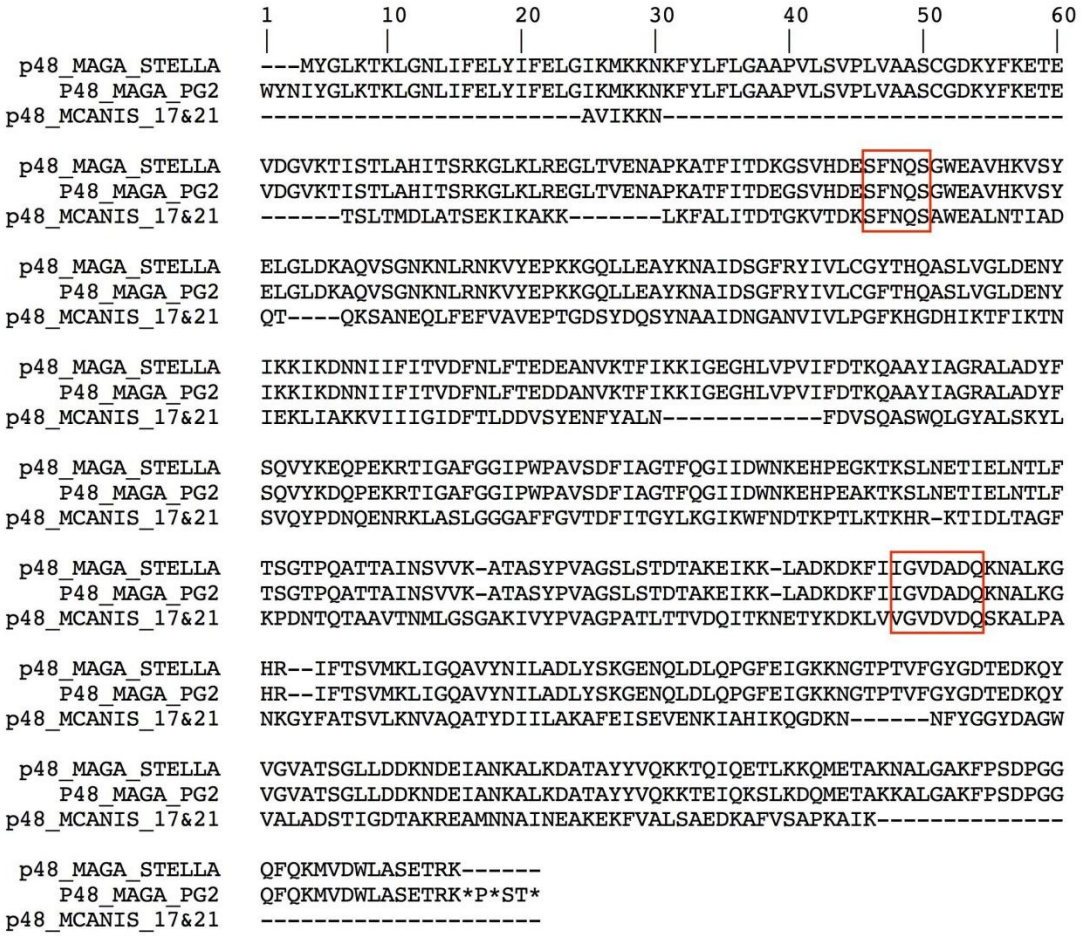

Figure 18: Comparison of p48 amino acid sequences of *M.agalactiae* (MA) PG2 and Stella, *M. canis* (MC) 17 and 21. MC p48 sequence contains two conserved motifs also present in MA p48 sequence – SFNQS and IGVD-DQ

3.3.1 p48 expression

The next step was to produce a recombinant p48 of MA and of MC. Due to differences between mycoplasmas and *E. coli* in codon usage, site directed mutagenesis was successfully performed to convert the TGA codon to TGG (Rosati et al., 2000). Three and four TGA codons were converted in the p48 gene of MC-21 and MA-PG2, respectively. The final product was efficiently cloned into expression vector pGex-6H. The GST/p48 fusion protein of both MA and MC was successfully expressed in soluble form in *E. coli* and migrated as <80 kDa in SDS-PAGE, corresponding to fusion of GST carrier (26 kDa) with P48 (48 kDa) (Figure 19, lane 3). The p48 protein was then purified by affinity chromatography (Smith and Johnson, 1988) and analyzed via SDS-PAGE (Figure 19, lane 4).

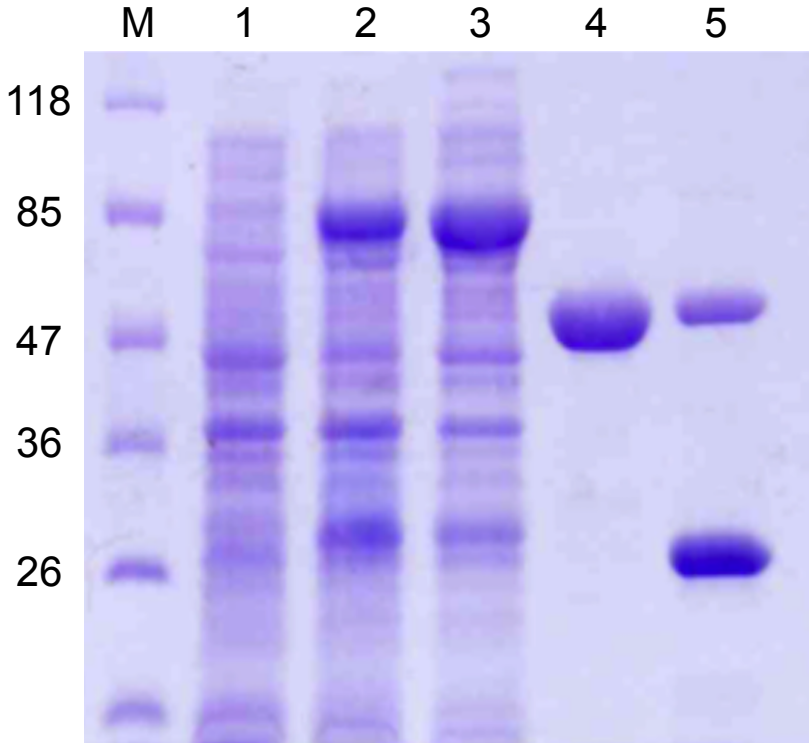

Figure 19: *M. canis* p48 analysis by SDS-PAGE. M: Marker; 1. Control - no induction with IPTG; 2. After induction with IPTG; 3. S0 - soluble fraction; 4. Elution p48; 5. Elution GST from matrix.

The two recombinant proteins were then used for rat immunization. The collected sera after the first and second booster were analyzed by ELISA, using the pre immune serum as negative control. Already after the second immunization high titers of binding antibodies were observed (data not shown). The anti-p48 polyclonal antisera obtained were employed in the development and standardization of colony blotting analysis.

Preliminary tests were performed, in order to evaluate whether there was any cross-reactivity between anti-p48-MC and anti-p48-MA. Spots of MC and different strains of MA (PG2, Stella and 5632) were plated on the same SP4 agar plate. A dot blot was performed on this plate using two different nitrocellulose membranes. The two membranes were incubated separately using anti-p48-MC and anti-p48-MA. All dilutions of anti-p48-MA (1:1000; 1:2000; 1:5000) reacted with the MA colonies, but not with the MC colonies. Instead, anti-p48-MC showed low levels of cross-reactivity using dilutions 1:1000 and 1:2000 (Figure 20); in fact, it was found to react with both MA and MC

colonies. Instead, using the dilution 1:5000 it reacted with MC colonies well, but poorly with MA colonies. Despite this low level of cross-reactivity, we decided nonetheless to apply this method to discriminate between transformed colonies, using the anti-p48-MC dilution 1:5000 only.

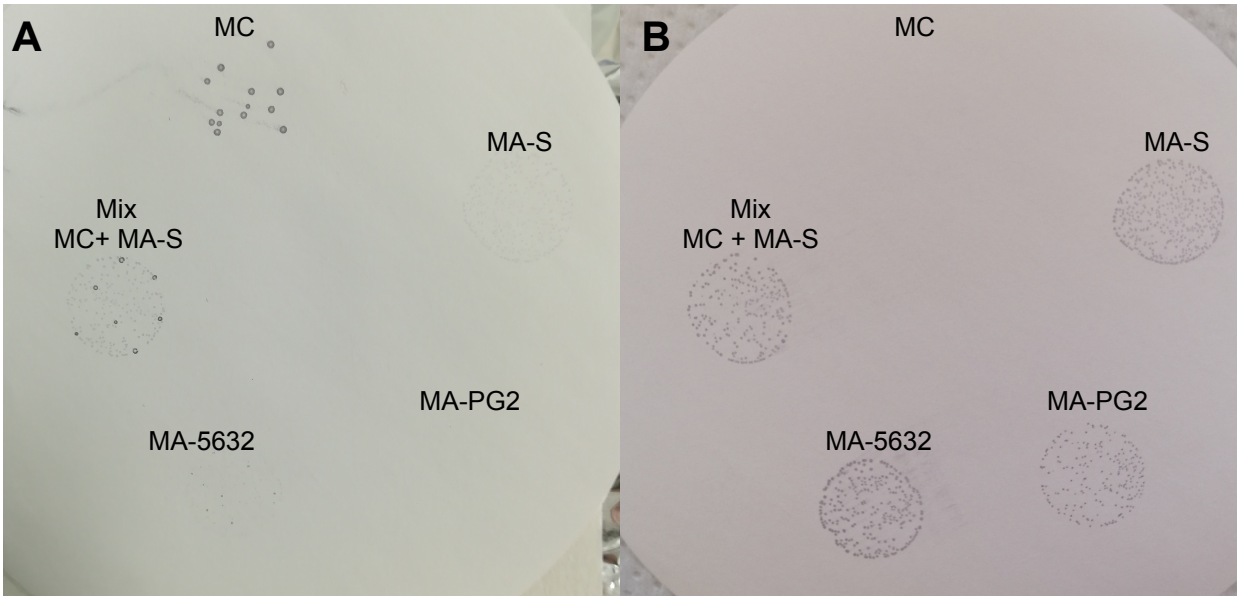

Figure 20: Colony blot analysis. Spots of *Mycoplasma canis* (MC), a mix of MC and *Mycoplasma agalactiae* strain Stella (MA-S) and different strains of *Mycoplasma agalactiae* PG2 (MA-PG2), MA-S and *Mycoplasma agalactiae* strain 5632 (MA-5632) were plated on the same SP4 agar plate. The colonies were blotted on two different nitrocellulose membranes. The membranes were incubated separately using a 1:1000 dilution of: A anti-p48-MC; and B) anti-p48-MA. In the spot containing the mix of MC and MA-S, the two different strains are distinguishable as the MC colonies are bigger than the MA-S colonies.

3.3.2 Donor DNA

All DNA inserts, containing the gene of interest flanked by two homology arms for HR, were successfully obtained using the concatenation method (Gibson et al., 2008). Then, the inserts were cloned into specific plasmids before the subsequent step to obtain ssDNA. During the cloning step, low efficiency was obtained for fragments bigger than 2 kb. We hypothesized that this low efficiency might be caused by the exposure of the DNA to UV light (Rand, 1996). To get around this problem,

instead of the Macherey-Nagel kit that involves brief UV exposure to visualize the DNA band, we applied a gel purification method that uses crystal violet to visualize DNA fragments as a thin blue band under normal light. This method causes less DNA damage and, as expected, drastically increased the cloning efficiency.

3.3.3 Mycoplasma transformation

Prior to MA transformation, a prerequisite step was to assess the MA sensitivity to antibiotics (tetracycline, gentamicin and puromycin) because transformation efficiency had always been tested using a plasmid that conferred AR to mycoplasmas. For broth dilution testing, the bacterium was spread in selective medium, and in medium with no antibiotic as control. The change in broth colour of the selective medium was compared with the control with no antibiotic to verify the bacterium growth (Hannan, 2000; CLSI (Clinical and Laboratory Standards Institute), 2011).

Antibiotic concentrations of 1 μg/ml tetracycline, 25 μg/ml gentamicin and 2.5 μg/ml puromycin were found to be completely inhibitory for MA-PG2, whereas 4 μg/ml tetracycline, 25 μg/ml gentamicin and 5 μg/ml puromycin were required to completely block MA-S.

Two different methods were used for mycoplasma transformation: electroporation, and PEG-8000 treatment.

3.3.3.1 Transformation via electroporation

With the aim of evaluating the transformation efficiency, several preliminary transformation experiments were performed with electroporation, using the plasmid pMM21-7 as the control for transformation efficiency. This plasmid contains the chromosomal replication origin of MA (*M. agalactiae ori*C) and the tetracycline resistance gene (*tet*M) (Chopra-Dewasthaly et al., 2005b).

Mycoplasma transformation is highly sensitive to the electroporation conditions used (Chopra-Dewasthaly et al., 2005a). Unfortunately, the manufacturer of the NucleofectorII device used in this study does not indicate the programs parameters to use; its set programs are indicated for different bacterial strains, and are not freely modifiable. Hence, all of the device's bacteria transformation programs were tested on MA using the pMM21-7 plasmid, but all transformation attempts failed. No

colonies were obtained even in the control condition in the absence of plasmid. This result implies that none of the programs tested are suitable for mycoplasmas transformation.

3.3.3.2 Transformation via PEG-8000

With the aim of optimizing the PEG-8000 method for MA transformation, we performed several preliminary experiments using the plasmids pMT85-gen and pMiniO-pur (kindly provided by the INRA/ENVT ENVT - Interaction Hôtes-Agents Pathogènes - research laboratories, in Toulouse - France) as controls for transformation efficiency. The pMT85-gen plasmid contains a modified version of transposon Tn*4001* (mini-Tn) that confers gentamicin resistance (Zimmerman & Herrmann, 2005). The pMiniO-pur plasmid, on the other hand, confers puromycin resistance. MA was successfully transformed with both pMT85-gen and pMiniO-pur using the PEG-8000 transformation protocol.

Several attempts were also made to transform MA using the CRISPR/Cas9 in combination with ss- and ds- donor DNA.

In an initial phase, MA-S and MA-PG2 were transformed using CRISPR in combination with HR-p48-canis donor DNA, as both ss- and dsDNA. Then, the transformed colonies were screened using colony blotting. Following MA transformation, the colony blotting screening process did not give any positive results. To investigate transformation efficacy further, we developed a specific PCR assay. To this end, we designed two primers: a forward primer complementary to a region upstream of the HR region of MA, and a reverse primer complementary to a region within the MC p48 gene. Thus, only in the case of efficient gene editing would a PCR product be obtained. The lysate of the transformed suspension was tested, and confirmed the negative result obtained by colony blotting.

Owing to these unsuccessful results, and considering that the selection of transformation events from the large number of non-transformed events is the most critical step in the production of genetically modified bacteria, an AR gene was added to the first donor construct to facilitate the screening process – even though the use of AR was initially excluded in order to avoid future problems related to the applicability of an eventual vaccine.

The new donor construct (HR-p48canis-PAC2), containing the MC-p48 gene fused with puromycin resistance (Pac2-Pur) gene, was used for the transformation of both MA-S and MA-PG2 in combination with the CRISPR system. It was possible to transform both strains with the same donor DNA because the two regions for HR are exactly the same in each strain.

Even using this strategy, no positive results were obtained. Nevertheless, a PCR assay was performed on the lysate of the transformed suspension, using a forward primer complementary to a

region upstream to the HR arm and a reverse primer complementary to the Pac2-Pur gene region. Once again, no positive results were obtained.

However, despite being a very important protein, considering that its expression is constant throughout all stages of infection (Rosati et al., 1999, 2000) as well as high compared with other Ma proteins, the role and the function of p48 has yet to be clarified. Although we chose an orthologous protein, the MC p48, which is likely to have the same function, it is possible that MA p48 is fundamental for MA survival. As a consequence, on the hypothesis that the CRISPR system introduced into the MA cells is able to cut the MA genome, it is possible that the p48 negative mutants are unable to survive.

For this reason, a third strategy was thus attempted. In the third strategy we changed the protein being replaced, and focused instead on another good marker of CA infection, the p40 protein. As the capability of p40 minus mutants to survive has been demonstrated (Skapski et al., 2011), we evaluated the feasibility of total MA p40 gene replacement with the Pac2-Pur gene. Sequence analyses were carried out to analyze the MA genome regions flanking the p40 gene. A third donor construct (HR-p40-PAC) containing the Pac2-Pur gene flanked by two regions for HR up- and downstream of the MA-PG2 p40 gene was used for MA-PG2 transformation with the CRISPR system.

After the transformation process, the MA suspension was spread on SP4 -agar plates added with puromycin. Interestingly, the growth was observed for some transformed colonies after 7 days of incubation at 37°C in contrast with the negative control (a spot of MA transformed without DNA was spread in the same plate) than never grew. Seven well-isolated colonies were picked and subjected to serial passage in selective SP4 medium using increasing concentrations of puromycin (5, 10 and 20 μg/ml). The colonies growth was observed at all the concentrations of puromycin tested, whereas the MA-PG2 reference strain growth was observed in medium without any antibiotic only.

PCR assays were performed on the colony lysates to verify the presence of the Pac2-Pur gene in the genome, and no positive results were obtained. Nevertheless, whole-genome sequencing of one out of the seven colonies obtained was performed to verify the presence of puromycin resistance or some mutation in the genome. The sequencing data failed to reveal the presence of any genome mutation as compared with the MA-PG2 reference strain.

One explanation may be that only a few bacteria within this population over-expressed the resistance factor into the medium, but to the benefit of the entire population.

Further studies are required to validate and investigate this last hypothesis and to analyze in more depth the antibiotic resistance of these colonies. Indeed, it is improbable that spontaneous AR was acquired by simple point mutation due to the puromycin mechanism of action which recruits the rRNA recognition elements used by all the various tRNAs in a cell (Algire et al., 2009).

3.3.4 Future developments

Next-generation sequencing technologies have become an important resource for all scientific disciplines, and can be exploited to investigate AR at the genome level, which is often not possible using PCR-based methods only (Pulido et al., 2013; Van Camp et al., 2020).

We performed RNAseq in just one of the seven colonies obtained (and no evidence of AR gene insertion was noticed), but it would be interesting to analyze the genome sequences of all the colonies in order to compare the genomes and analyze the differences in genome mutations between the seven colonies. In addition, to better characterize the AR of these colonies, we could study the changes in gene expression of the isolated colonies upon drug treatment compared with the negative control condition (MA transformed without DNA) which fails to grow in antibiotic-treated medium (Suzuki et al., 2014; Darnell et al., 2019).

Another question to resolve is whether the CRISPR system as an RNP complex can be used for Mycoplasma transformation. Indeed, one important drawback of the Cas9-RNP system for mycoplasma transformation is thought to arise from the high level of nucleases present on the mycoplasma membrane. These proteins play an important role in mycoplasmas pathogenicity as virulence factors (Paddenberg et al., 1998; Wang et al., 2001; Sirand-Pugnet et al., 2007). However, considering that Mycoplasmas are unable to synthesize de novo nucleic acid precursors, because they lack the biosynthetic machinery to do so (Paulsen et al., 2000), external membrane-associated nuclease activity may also play an important role in the processing and importing of nucleosides derived from nucleic acids included in culture media and in host tissue. Membrane nucleases have been found in many mycoplasmal species (Minion et al., 1993; Li et al., 2010; Somarajan et al., 2010), including MA (Cacciotto et al., 2013). Consequently, we can hypothesize that these nucleases may contribute to hindering or lowering the efficiency of transformation of mycoplasma with exogenous DNA. So, important parameters that could be further investigated in the future include: incrementation of the Cas9-RNP and the donor DNA concentrations, up to the limit at which they becomes toxic; RNP and DNA protection, for example using the wide range of systems for delivering the Cas9-RNP and the donor DNA into cells (Kim et al., 2020). In the future, it may be interesting to apply and optimize different delivery systems for mycoplasmas, such as liposomes, in order to introduce and, at the same time, to protect the Cas9-RNP and the donor DNA.

Furthermore, a future strategy for the genome editing of MA could involve the identification of new gene targets to replace using the same approach. Indeed, this study focussed on two good markers of infection, p40 and p48 (Rosati et al., 2000; Fleury et al., 2002), but other MA lipoproteins could be deleted or replaced for the development of marker vaccines, such as P80 (Tola et al., 2001) and P30 (Fleury et al., 2001).

Finally, another future development of this study to evaluate the applicability of the CRISPR system in general, and of the RNP complex in particular, for editing the MA genome could be to investigate the use of a mycoplasma-derived CRISPR system. A recent study demonstrated the possibility of using the endogenous *Mycoplasma gallisepticum* CRISPR/Cas9 system to induce precise mutations in the genome of this pathogen (Mahdizadeh et al., 2020).

Analysis of the MA genome did not reveal the presence of CRISPR arrays (http://crispr.i2bc.paris-saclay.fr/) that could be used as an endogenous CRISPR system. However, considering the similarities in DNA repair mechanisms between different mollicutes, it would be interesting to investigate the use of the *M. gallisepticum* engineering CRISPR/cas9 system developed by Mahdizadeh and colleagues (Mahdizadeh et al., 2020) to mutagenize and then edit the MA genome.

This research was a preliminary study to evaluate the feasibility of the CRISPR/Cas9 system as an RNP complex for the genome editing of Mycoplasmas. Due to the well-known difficulties in the genome editing and manipulation of Mycoplasmas, we were met with many obstacles, and were regrettably unable to clarify the potential of this method in this microorganism. Certainly, the future studies described above might help us to understand why the experiments in this research did not work, and they will be fundamental to complete in order to continue our investigations into the genome editing of these unique and fascinating microorganisms, the smallest and simplest self-replicating bacteria known to man.

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