universität wien

DISSERTATION

Characterization of DNA rearrangements within the *vpma* multigene locus of *Mycoplasma agalactiae*

angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr. rer. nat.)

Verfasser:	Mag. rer. nat. Stefan Czurda
Dissertationsgebiet:	A 091 441 Doktoratstudium der Naturwissenschaften, Studienzweig: Genetik - Mikrobiologie
Betreuer:	O. Univ. Prof. Dr. Dr. habil. Renate Rosengarten

Wien, im August 2010

Durchgeführt am Institut für Bakteriologie, Mykologie und Hygiene der Veterinärmedizinischen Universität Wien

Zusätzliche Betreuung:

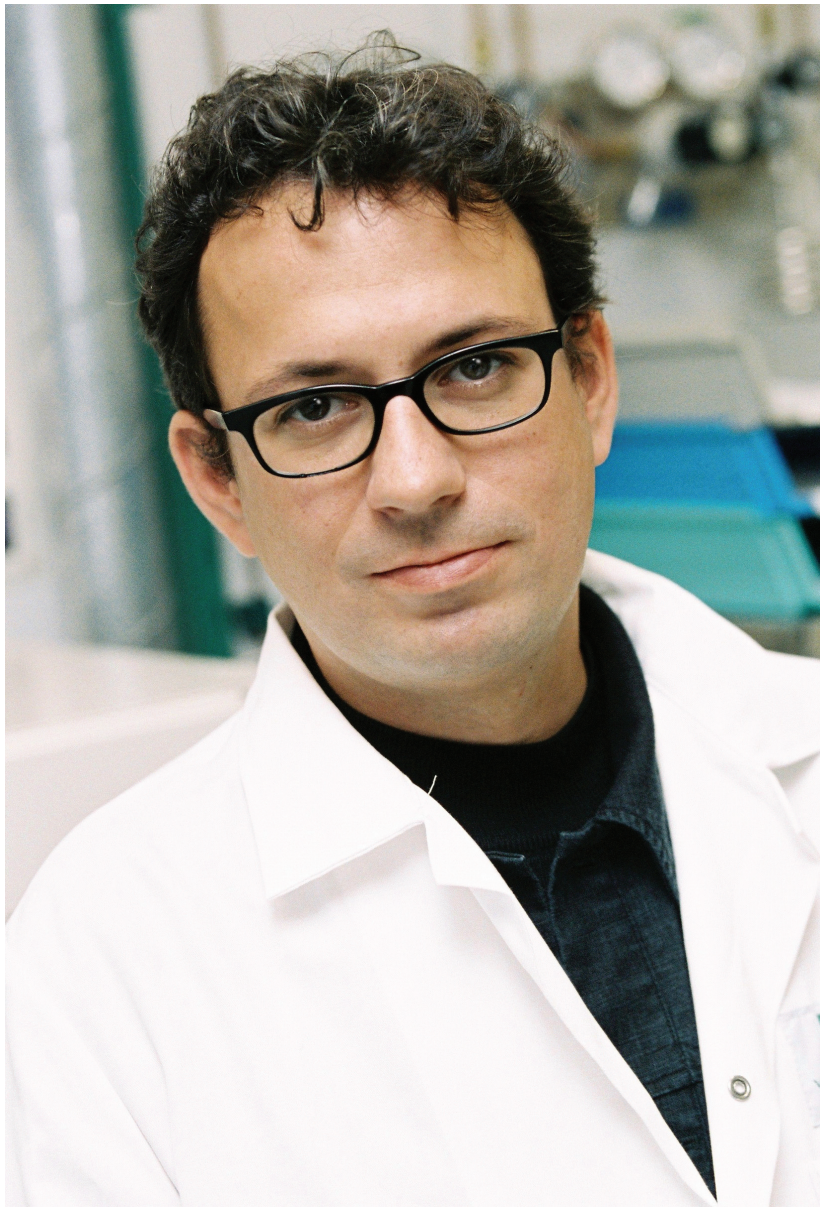
Innerhalb der Mykoplasmen-Forschungsgruppe am Institut für Bakteriologie, Mykologie und Hygiene der Veterinärmedizinischen Universität Wien

Dr. Wolfgang Jechlinger

Dr. Rohini Chopra-Dewasthaly

Administrative Betreuung an der Universität Wien

Prof. Dr. Angela Witte



Für Wolfi

TABLE OF CONTENTS

1 **ABBREVIATIONS** 7

2 **ABSTRACT** 8

2.1 Abstract (English) 8

2.2 Abstract (German) 10

3 **INTRODUCTION** 12

3.1 General characterization of the genus *Mycoplasma* 12

3.2 Antigenic variation in *Mycoplasma* species 13

3.3 *Mycoplasma agalactiae* 14

3.3.1 Current research on *M. agalactiae* 14

3.3.2 The Contagious Agalactia syndrome 15

3.3.3 The *vpma* multigene locus 17

3.3.4 Molecular mechanism of Vpma antigenic variation in *M. agalactiae* 18

3.4 Site-specific recombination 19

3.4.1 General characteristics of site-specific recombination 19

3.4.2 The λ-integrase family of site-specific recombinases 20

3.4.3 Recombination sites (RS) involved in site-specific recombination 20

3.4.4 *Mycoplasma* site-specific recombinases 21

3.5 Work description 21

4 **REFERENCES INTRODUCTION** 23

5 **RESULTS AND PUBLICATIONS** 27

5.1 Publication: Xer1-Mediated Site-Specific DNA Inversions and Excisions in *Mycoplasma agalactiae* 27

5.2 Collection of Data: Xer1-independent Mechanisms of Vpma Phase Variation are Triggered by the Presence of Vpma-specific Antibodies 64

6 **APPENDIX** 89

6.1 Solutions and Media 89

6.1.1 Media, media additives and antibiotics 89

6.1.2 Southern blot analysis 91

6.1.3 Agarose gel electrophoresis 92

6.1.4 DNA isolation 93

6.1.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 94

6.1.6 Western blot and colony blot analysis 95

6.2 Plasmid constructs 96

6.2.1 Symbols and abbreviations 96

6.2.2 Construction of plasmid pBADRS21x2 97

6.2.3 Construction of plasmid pBADXerRS21 98

6.2.4 Construction of plasmid pBADXerRS21x2 99

6.2.5 Construction of plasmid pBADXerIR200Y 100

6.2.6 Construction of plasmid pBADXerDR200Y 101

6.2.7 Construction of plasmid pBADXerIR21Y 102

6.2.8 Construction of plasmid pBADXerIR31Y3' 103

6.2.9 Construction of plasmid pBADXerIR56Y3' 104

6.2.10 Construction of plasmid pBADXerIR111Y 105

6.2.11 Construction of plasmid pBADXerIR57Y5' 106

6.2.12 Construction of plasmid pBADXerIR200Y/184U 107

6.2.13 Construction of plasmid pBADXerIR200Y/56X5' 108

6.2.14 Construction of plasmid pBADXerIR53U5'/57Y5' 109

6.2.15 Construction of plasmid pIL 110

6.2.16 Construction of plasmid pILDR 111

6.3 *vpma* locus of X-EV, PLM18 and PLM16 – annealing sites of sequencing primers 112

ACKNOWLEDGEMENTS 113

CURRICULUM VITAE 114

1 Abbreviations

ATP	adenosine triphosphate
bp	base pairs
CA	Contagious Agalactia
CFU	colony forming unit
DIG	digoxigenin
DNA	deoxyribonucleic acid
ddH_2O	double distilled water
dNTP	deoxynucleotide triphosphate
E.	*Escherichia*
IS	insertion sequence
kB	kilo base
LB medium	Luria-Bertani medium
LC	large colony type
M.	*Mycoplasma*
mc	mini circle
mp	mini plasmid
OD	optical density
op	original plasmid
ORF	open reading frame
pAb	polyclonal antibody
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLM	phase-locked mutant
R-H-R-Y	arginine-histidine-arginine-tyrosine
RNA	ribonucleic acid
RS	recombination site
sc	supercoiled
tRNA	transfer ribonucleic acid
TBS	tris buffered saline
U	unit
UTR	untranslated region
X-EV	VpmaX expressing escape variant
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

2 Abstract

2.1 Abstract (English)

Mycoplasma agalactiae, a cell wall-less bacterium belonging to the class *Mollicutes*, is considered the main etiological agent of the syndrome Contagious Agalactia, which primarily causes mastitis, arthritis and conjunctivitis in sheep and goats. Antigenic diversity in *M. agalactiae* is governed by DNA rearrangements within the *vpma* multigene locus encoding the Vpma family of variable surface lipoproteins. Six *vpma* genes were shown to undergo high-frequency phase variation by site-specific DNA inversions mediated by a site-specific recombinase (Xer1) encoded adjacent to the *vpma* genes. Disruption of the *xer1* recombinase gene has demonstrated that the Xer1 recombinase is essential for site-specific recombination. In the so-called phase-locked *xer1* mutants (PLMs) further *vpma* switching by site-specific DNA inversions was abolished and cells were locked in an expression phase where only one *vpma* gene is steadily expressed. In this study, recombination experiments in *Escherichia coli* demonstrated that a putative 21-bp recombination site (RS) located in the 5' UTR of all six *vpma* genes is indeed involved in Xer1-mediated recombination. Furthermore, adjacent nucleotides flanking this conserved 21-bp region were shown to positively influence the recombination reaction. The orientation of the RS was crucial for the outcome of the recombination reaction. Recombination between inverted repeated RS led to inversions, whereas direct repeated alignment of the RS resulted in excisions. Excision events were also successfully demonstrated under native conditions in *M. agalactiae*, using a newly developed assay based on the *lacZ* reporter gene, which was the first application of this reporter system in *M. agalactiae*.

Beside site-specific Xer1-mediated recombination, novel molecular mechanisms resulting in Vpma surface variation in the absence of the Xer1 recombinase were demonstrated. In another ongoing project PLMs lacking the *xer1* gene, and therefore steadily expressing only a single Vpma product, were used in animal infection studies. Interestingly, PLMs isolated from the animals displayed a new Vpma phenotype. Molecular characterization of selected PLM switchover clones revealed that complex DNA rearrangements including generation of hybrid *vpma* genes, as well as gene duplications and deletions were triggered in the *vpma* locus, most likely due to the host immune

pressure. Similar Xer1-independent Vpma variation could be demonstrated during in vitro immune pressure assays using Vpma-specific antibodies. These results clearly demonstrate that phase variation of Vpma surface proteins in *M. agalactiae* is necessary to survive in the immunocompetent host and provide important data for studying similar antigenic variation systems in other pathogenic *Mycoplasma* species.

2.2 Abstract (German)

Mycoplasma agalactiae, ein Vertreter der taxonomischen Klasse *Mollicutes*, stellt als Erreger der Infektiösen Agalaktie der Schafe und Ziegen ein wichtiges pathogenes Bakterium dar. Die klinische Symptomatik der Krankheit ist charakterisiert prädominant durch Mastitis, Arthritis und Konjunktivitis. Innerhalb des *M. agalactiae*-Genoms konnte ein einer Pathogenitätsinsel ähnlicher Genlokus identifiziert werden, welcher die wichtigsten immunodominanten Oberflächenproteine, die sogenannten Vpmas (variable proteins of *M. agalactiae*) kodiert. Durch ortsspezifische DNA-Umlagerungen, die durch das Xer1-Protein katalysiert werden, welches in direkter Nachbarschaft zu den *vpma*-Genen kodiert wird, wird die Expression der Vpma-Proteine mit einer ungewöhnlich hohen Frequenz variiert. Durch gezielte Disruption des *xer1*-Gens konnte gezeigt werden, dass die Xer1-Rekombinase den essentiellen Faktor für ortsspezifische DNA-Inversionen innerhalb des *vpma*-Genlokus darstellt. In diesen sogenannten *xer1*-phase-locked mutants (PLMs) finden daher keine spezifischen DNA-Umlagerungen mehr statt, da die Zellen in einer bestimmten Vpma-Expressionsphase arretiert bleiben. Die vorliegende Arbeit beinhaltet neue Daten bezüglich dieses Antigenvariationssystems von *M. agalactiae*.

Rekombinationsexperimente in *Escherichia coli* zeigten, dass vorausgesagte Rekombinationsstellen (RS) von 21 bp, welche sich in der 5' UTR aller sechs *vpma*-Gene befinden, tatsächlich in Xer1-katalysierten Rekombinationsereignissen involviert sind. Angrenzende Nukleotide konnten die Rekombinationsraten positiv beeinflussen. Zusätzlich konnte gezeigt werden, dass die Anordnung dieser RS für das Ergebnis der Rekombinationsereignisse eine essentielle Rolle spielt. Während invertiert angeordnete RS zu Inversionen führten, kam es an RS in direkter Abfolge zu Exzisionsereignissen. Zusätzlich konnten ortsspezifische Exzisionen im Genom von *M. agalactiae* mithilfe eines neu entwickelten *lacZ*-Reportersystems nachgewiesen werden.

Neben ortsspezifischen DNA-Umlagerungen konnten neue molekulare Mechanismen der Vpma-Antigenvariabilität von *M. agalactiae* gezeigt werden, die speziell in der Abwesenheit der Xer1-Rekombinase stattfinden. Dazu wurden PLMs, die nur ein bestimmtes Vpma-Genprodukt exprimieren, im Tierversuchsmodell verwendet. Überraschenderweise zeigten jene PLMs, die aus den Tieren

reisoliert wurden, einen neuen Vpma-Phenotyp. Die molekulare Analyse durch DNA-Sequenzierung zeigte, dass komplexe DNA-Umlagerungen, wie beispielsweise die Entstehung chimärer *vpma*-Gene bzw. Genduplikation und -deletionen, durch den Selektionsdruck des Immunsystems in den Reisolaten stattgefunden haben. Ähnliche Ergebnisse wurden auch in einem neu entwickelten in vitro-Experiment unter Selektionsdruck in Gegenwart von Vpma-spezifischen Antikörpern erhalten. Diese neuen Daten unterstreichen die Bedeutung der Antigenvariation als wichtige Überlebensstrategie des Erregers *M. agalactiae* in seinem Wirt und repräsentieren eine wichtige Grundlage für die Untersuchung ähnlicher Systeme in anderen pathogenen Mykoplasmenarten.

3 Introduction

3.1 General characterization of the genus *Mycoplasma*

The genus *Mycoplasma* belongs to the taxonomic class *Mollicutes* representing an important group of bacteria that are widespread in nature as commensals and pathogens of plants, animals and humans. *Mollicutes* can be taxonomically distinguished from other bacteria by the total lack of a peptidoglycan cell wall. Therefore, they are completely resistant to antibiotics targeting cell wall synthesis such as beta-lactams (Baseman and Tully, 1997; Razin et al., 1998).

Due to their small size and limited genomic capacity, *Mycoplasma* species are commonly described as prototypes of a minimal organism. With remarkably reduced cell sizes ranging from 0.3 μm to 0.8 μm, they constitute the smallest autonomously replicating bacteria. Genome sizes vary from 580 kb (*M. genitalium*) (Fraser et al., 1995) to 1,340 kb (*M. mycoides* subsp. *mycoides* LC, which is now reclassified as *M. mycoides* subsp. *capri*) (Manso-Silvan et al., 2009) and are the smallest of all free-living organisms (Razin, 1997). Additional features of a typical *Mycoplasma* genome include the characteristically low GC-content (25-33 mol %) (Razin et al., 1998) and the use of a typical UGA stop codon as a tryptophan codon (Osawa et al., 1992).

Mollicutes are thought to have evolved from low GC Gram-positive bacteria (*Firmicutes*) by a so-called regressive evolution (Woese et al., 1980). Consequently, to favor a small genome, most genes for metabolic pathways are missing, which reflects their host-dependent lifestyle. The primary habitats of human and animal *Mycoplasma* species are the mucous surfaces of the respiratory and urogenital tracts, the eyes, mammary glands and joints. Infections are usually characterized by clinical symptoms like pneumonia, urethritis, conjunctivitis, mastitis and arthritis (Razin et al., 1998). Due to their fastidious nutritional requirements they usually exhibit relatively strict host and tissue specificity (Rottem and Naot, 1998). Infections with pathogenic *Mycoplasma* species are usually mild but difficult to eradicate and follow a chronic course. For this reason, *Mycoplasma* species are considered very close to the concept of the ‘ideal parasites’ which are properly adapted and rarely kill their hosts (Razin et al., 1998; Razin, 1999).

Despite their biological attraction as minimal organisms and their medical and economical importance as pathogens of human and animals, *Mollicutes* are also well known contaminants of eukaryotic cell line cultures, which cause major problems in research, diagnosis and biotechnological production (McGarrity et al.,1992; Rawadi and Dussurget, 1995).

3.2 Antigenic variation in *Mycoplasma* species

The term 'antigenic variation' refers to the ability of a microbe to change the antigenic character of its cell surface components that are the major targets of the host immune system and consequently allow effective avoidance of immune recognition (Razin et al., 1998). In the absence of a cell wall, the cytoplasmic membrane is the key element for interaction with the immune system. In order to establish chronic infections and to persist in the host, *Mycoplasma* species are equipped with sophisticated molecular systems that promote variation in expression and structure of abundant surface lipoproteins (Wise, 1993; Rottem and Naot, 1998). Despite the limited coding capacity of *Mycoplasma* genomes, the number of genes involved in antigenic variation systems is unexpectedly large (Razin et al., 1998). Variable surface components can be either encoded by single genes or by multigene families (Citti et al., 2005). Several *Mycoplasma* species have been shown to express surface antigens that vary in expression and/or undergo spontaneous changes in size resulting in a remarkably high antigenic repertoire on the cell surface (Citti and Rosengarten, 1997; Rosengarten et al., 2000).

Phase variation is a mode of gene regulation that produces cells within a clonal population that either express (phase ON) or do not express (phase OFF) a particular antigen and is usually reversible (van der Woude and Baumler, 2004). Several genetic mechanisms associated with phase variation have been described in different *Mycoplasma* species and are governed by minor or major changes in the chromosomal DNA of the organism (Citti and Rosengarten, 1997).

Surface antigens may also undergo spontaneous size variation, which affects their structure, in most cases by altering the length of their carboxyl-terminal regions (Citti et al., 2005). Antigen-encoding genes frequently include multiple tandem repetitive domains. These modules are hot spots for mutations, which expand or contract these repetitive sequences resulting in the expression of different size variants of a particular antigenic protein (Razin et al., 1998).

The first fully described system of antigenic variation in the genus *Mycoplasma* was the Vlp system of the porcine pathogen *M. hyorhinis* (Rosengarten and Wise, 1990; 1991). The *vlp* gene family consists of a set of related genes encoding surface lipoproteins, which are switched ON and OFF at high frequencies. Regulation of phase variation occurs at the transcriptional level, where spontaneous mutations in the poly-(A) sequence of the *vlp* promoter are responsible for ON and OFF switching of the *vlp* genes. The insertion or deletion of a single adenine nucleotide is sufficient to change the expression of a *vlp* gene (Yogev et al., 1991). Moreover, these membrane proteins may also undergo size variation by changing the number of repetitive elements in the C-terminal region, resulting in elongated or shortened Vlp molecules (Rosengarten and Wise, 1991). As each phase variation can independently be superimposed by size variation, there is a great variety of possible antigenic combinations on the surface of *M. hyorhinis* (Rosengarten and Wise, 1991).

Variable expression of surface lipoproteins can also occur by a so-called 'cut and paste' mechanism (Citti et al., 2005). The *vsa*, *vsp* and *vpma* families of *M. pulmonis*, *M. bovis* and *M. agalactiae*, respectively, have been documented to use site-specific recombination to alternatively switch between ON and OFF expression phase of a particular surface lipoprotein (Ron et al., 2002).

3.3 *Mycoplasma agalactiae*

3.3.1 Current research on *M. agalactiae*

During the last decade, a variety of novel tools and information have accumulated to greatly enhance the knowledge on the molecular biology and pathogenicity mechanisms of the ruminant pathogen *M. agalactiae*. The discovery of the *vpma* multigene family (see section 3.3.3.) constitutes an important start point for studying molecular pathogenicity of *M. agalactiae* (Glew et al., 2000). The *vpma* genes have been shown to undergo phase variation by site-specific recombination (see section 3.3.4), which resembles a common mechansim of antigenic variation exhibited by many other *Mycoplasma* species (Glew et al., 2002; Ron et al., 2002; Flitman-Tene et al., 2003; Horino et al., 2003). The previous lack of tools to genetically manipulate *M. agalactiae* has so far hampered more refined studies to assess the exact function of Vpmas in *M. agalactiae* infection and disease. However, this has changed since the establishment of the first successful transformation protocol, which formed an important step towards

the genetic manipulation of *M. agalactiae* (Chopra-Dewasthaly et al., 2005). This was followed by the construction of *E. coli* - *M. agalactiae* shuttle vectors that were instrumental in the first targeted gene disruption via homologous recombination (Chopra-Dewasthaly et al., 2005; 2008). Inactivation of the *xer1* recombinase gene by homologous recombination (see section 3.3.4) could demonstrate that the Xer1 recombinase is indeed the factor involved in *vpma* gene rearrangements leading to phase variation of the corresponding six Vpma surface lipoproteins in the type strain PG2 (Chopra-Dewasthaly et al., 2008). Also, the construction of specific Vpma phase-locked mutants (PLMs) by Xer1 disruption greatly enhanced the ability to evaluate the role of the Vpma lipoproteins during *M. agalactiae* infection studies.

Furthermore, the genome sequence of the *M. agalactiae* type strain PG2 is available since 2007 and has provided valuable information (Sirand-Pugnet et al., 2007). It constitutes a typical *Mycoplasma* genome with a small size (877 kb), low GC content (29,7%) and high gene compaction. Comparative analysis of the *M. agalactiae* genome with that of other members of the class *Mollicutes* revealed that about 18% genes, several of which encode lipoproteins that are involved in interactions with the host, have been horizontally transferred from *Mycoplasma* species of the mycoides cluster that are phylogenetically different but share with *M. agalactiae* common ruminant hosts (Sirand-Pugnet et al., 2007). This large scale acquisition of foreign genes represents an important evolutionary aspect that stands in contrast with the generally accepted scenario that gene loss is the main driving force of *Mycoplasma* evolution (Sirand-Pugnet et al., 2007).

In a recent genomic study, an extended repertoire of *vpma* genes was shown to be distributed over two distinct loci in the *M. agalactiae* field strain 5632 as compared to the single *vpma* locus found in the type strain PG2 (Nouvel et al., 2009).

3.3.2 The Contagious Agalactia syndrome

M. agalactiae is the main etiological agent of the syndrome Contagious Agalactiae (CA) of sheep and goats and represents one of the most important diseases in small ruminants caused by *Mycoplasma* species (Bergonier et al., 1997). The pathogen was isolated by Bride and Donatien in 1923 as the second known *Mycoplasma* species (Madanat et al., 2001). The disease was first described by Metaxa

(Italy) in 1816 and was given the name CA by Brusasci in 1871 (Bergonier et al., 1997; Madanat et al., 2001). CA is mainly characterized by the clinical symptoms of mastitis in lactating females. Males, young animals and non-lactating females suffer from arthritis, conjunctivitis and respiratory problems (Bergonier et al., 1997; Madanat et al., 2001; Corrales et al., 2007). Although *M. agalactiae* is considered the 'classical' agent of CA, yet *M. capricolum* subsp. *capricolum*, *M. putrefaciens, M. mycoides* subsp. *mycoides* LC, which is now reclassified as *M. mycoides* subsp. *capri*, can also produce a clinically similar disease (Bergonier et al., 1997; Manson-Silvan et al., 2009).

At present, CA is notifiable to the World Organization for Animal Health and occurs mainly in the Mediterranean area, but also on the Balkan peninsula, in western Asia and in northern, central and eastern Africa, leading to considerable economic losses mainly due to reduction in milk yields, but also due to pneumonia, abortion and in rare cases mortality (Nicholas, 2002; Corrales et al., 2007).

Under natural conditions, *M. agalactiae* transmission occurs through direct contact between infected and healthy animals. Young animals are commonly infected while suckling contaminated colostrum or milk. Also, infection can occur via contaminated milking equipment or the hands of the milker (Madanat et al., 2001).

The clinical diagnosis of CA is based on the presence of typical clinical signs related to the disease, which are rarely characteristic, so isolation and identification of the infectious agent is essential to confirm disease outbreaks (Nicholas, 2002). The most suitable samples for diagnosis are milk, mastitic secretion, joint fluid or eye swabs (Madanat et al., 2001; Nicholas, 2002) which are used for cultivation in liquid or on solid media (Lambert, 1987). Also, many serological tests have been developed to detect CA, which are especially useful for epidemiological studies, but all share difficulties related to cross-reactivity between closely related species (Corrales et al., 2007). These difficulties can be overcome by the use of molecular techniques and monoclonal antibodies (Bergonier et al., 1997; Corrales et al., 2007).

Controlling the disease by antibiotic therapy is usually ineffective, because such therapy reduces the clinical symptoms but fails to eliminate the causative agent and therefore promotes the carrier state (Nicholas, 2002). Prevention of CA caused by *M. agalactiae* by vaccination is widely used in the Mediterranean countries of Europe and the Middle East. Live vaccines have been used for many years

in Turkey and have been reported to provide better protection in ewes and their lambs than inactivated vaccines, but may produce a transient infection with shedding of the pathogen. In Europe, where live vaccines for *M. agalactiae* are not acceptable, attention has focused on the use of inactivated organisms. Globally, no single vaccine has been adopted and no standard method of evaluation and preparation has been applied (Nicholas et al., 2009).

3.3.3 The *vpma* multigene locus

Within the genome of the *M. agalactiae* type strain PG2 a multigene locus was identified that contains six distinct but related genes that encode the major antigenic surface lipoproteins, the Vpmas (variable proteins of *M. agalactiae*) (Glew et al., 2000; 2002; Flitman-Tene et al., 2003). DNA sequencing and protein analysis revealed that all *vpma* genes (*vpmaU* to *vpmaZ*) share a highly conserved 5' untranslated region (UTR), and a common leader sequence which ends in protease cleaving site. Based on their N-terminal sequence, *vpma* genes can be divided into two homology groups. Group one includes *vpmaW*, *vpmaX* and *vpmaY* and is characterized by a short region of high identity after the leader sequence, whereas group two consists of *vpmaV*, *vpmaU* and *vpmaZ* sharing common regions. Additionally, repetitive domains were identified within *vpma* genes, which are either fully or partially repeated within one gene, but may also form a part of a different *vpma* gene. A unique promoter is also present in the locus, which enables the expression of the downstream *vpma* gene (Glew et al., 2002).

However, two ORFs (ORF2' and *xer1*) unrelated to the *vpma* genes were identified at one end of the *vpma* locus of PG2. Whereas the function of ORF2' is unknown, the other ORF, designated as *xer1*, was shown to have significant homology to genes belonging to the λ-integrase family of site-specific recombinases which are characterized by an invariant tetrad of the amino acids R-H-R-Y that are involved in catalysis (Glew et al., 2002). It was shown that the Xer1 recombinase is responsible for phase variation of the *vpma* genes (Chopra-Dewasthaly et al., 2008). A tRNA gene was found between the two ORFs and appears to be unrelated to genes from *Mycoplasma* species and may constitute a hotspot for integration of foreign DNA (Glew et al., 2002). The presence of possible virulence genes (*vpma* mulitgene family), a 'mobility gene' (*xer1*) and a tRNA gene (tRNA-lys) suggests that the

vpma locus is a 'pathogenicity island-like' locus that constitutes a region of a chromosome encoding putative pathogenicity related genes (Hacker et al., 1997; Glew et al., 2002).

3.3.4 Molecular mechanism of Vpma antigenic variation in *M. agalactiae*

It has been demonstrated that the *vpma* genes undergo phase variation at high frequencies in vitro (10^{-2} to 10^{-3} per cell per generation) in the *M. agalactiae* type strain PG2 (Glew et al., 2000; Chopra-Dewasthaly et al., 2008). Only one *vpma* gene is transcribed at a given time from a single promoter present in the *vpma* locus, while all other *vpma* genes remain silent (Glew et al., 2002). The Xer1 recombinase encoded adjacent to the *vpma* genes was predicted to mediate DNA inversions responsible for switching the promoter from an active *vpma* gene to a silent one resulting in alteration of *vpma* expression. Sequence alignment of the *vpma* genes identified a conserved 21-bp region within the 5' UTR of all *vpma* genes that was thought to constitute the recombination site (RS) involved in Xer1-mediated inversions (Glew et al., 2000; Flitman-Tene et al., 2003). Disruption of the *xer1* gene in PG2 by homologous recombination proved that the Xer1 recombinase is indeed responsible for Vpma phase variation in *M. agalactiae* (Chopra-Dewasthaly et al., 2008). In the *xer1* knock-out mutants of PG2, further Vpma switching is abolished, and expression is blocked in a phase where only one *vpma* gene is steadily expressed. Complementation of the wild-type *xer1* gene in these phase-locked mutants (PLMs) restored Vpma phase variation (Chopra-Dewasthaly et al., 2008).

The *vsa* family of the murine pathogen *M. pulmonis* and the *vsp* family of the bovine pathogen *M. bovis*, the two phylogenetically close relatives of *M. agalactiae*, use very similar molecular mechanisms for generating surface diversity by DNA rearrangements (Ron et al., 2002). In both systems, as in the *vpma* locus, ON and OFF switching is mediated by a site-specific recombinase of the λ-integrase family and both contain a single promoter that drives the expression of only one gene within the multigene locus. However, the HvsR site-specific recombinase of *M. pulmonis* is known to exhibit a dual substrate specificity mediating ON and OFF switching of *vsa* genes, and also causing DNA inversions in the *hsd* locus resulting in the phase variable production of restriction enzymes with different sequence specificity (Sitaraman et al., 2002).

DNA rearrangements also govern phase variation of the Mpl lipoproteins that are encoded by the *mpl* gene family found as three different gene clusters in the human pathogen *M. penetrans* (Neyrolles et al., 1999; Röske et al., 2001; Sasaki et al., 2002). However, this molecular switch differs from the *vsa* and *vsp* families of *M. pulmonis* and *M. bovis* respectively, as each *mpl* gene has its own invertible promoter and can be individually switched ON and OFF, resulting in a large number of possible Mpl surface configurations (Horino et al., 2003).

Recent studies on the *M. agalactiae* field strain 5632 revealed a much higher repertoire of *vpma* genes compared to the type strain PG2 (Nouvel et al., 2009). A total of 23 *vpma* genes are distributed in two distinct loci, both containing a *xer1* recombinase gene and a single promoter allowing concomitant Vpma switching and expression in these loci. Several of these newly described *vpma* genes in strain 5632 display chimeric structures that are thought to be generated by homologous recombination events between different *vpma* genes, gene duplication, and/or insertion-deletion of repeated motives (Nouvel et al., 2009).

3.4 Site-specific recombination

3.4.1 General characteristics of site-specific recombination

DNA rearrangements in bacteria may either occur by general recombination between homologous DNA sequences or by a variety of specialized recombination events, such as site-specific recombination that is focused at specific sites in the genome. In a typical site-specific recombination reaction, a recombinase enzyme acts at well defined RS that are necessary for recombination and that contain the point of strand exchange. Site-specific recombination systems are widespread among bacteria and, depending on the orientation of the participating RS, serve various biological functions (see section 3.4.3) (Nash, 1996; Hallet and Sherratt, 1997). Important features of site-specific recombination are that (i) the recombination reaction may occur in the absence of replication, (ii) energy co-factors (such as ATP) are not required, (iii) and strand exchange is completed without any DNA synthesis or degradation. Depending on the catalytic mechanism used during recombination, site-specific recombinases can be separated into two distinct groups, which are referred to as the the λ-integrase family and the resolvase/invertase family (Nash, 1996; Hallet and Sherratt, 1997).

3.4.2 The λ-integrase family of site-specific recombinases

Members of the λ-integrase family of site-specific recombinases are characterized by four strongly conserved amino acid residues (R-H-R-Y) within the C-terminal half of the protein. Except for the R-H-R-Y tetrad, members of the λ-integrase family show only little sequence similarity with one another (Nunes-Düby et al.,1998). The recombination reaction proceeds via sequential strand exchange by formation and resolution of a Holliday structure intermediate during which the recombinase is covalently linked to the DNA by the tryosine residue (Hallet and Sherratt, 1997). This feature distinguishes the λ-integrase family from members of the resolvase/invertase family, which use a different mechanism for recombination (Hallet and Sherratt, 1997; Smith and Thorpe, 2002). Well studied members of this family include the lamdba Int protein, the Cre protein of the phage P1 and the XerC and XerD recombinases of *E. coli* amongst many others.

3.4.3 Recombination sites (RS) involved in site-specific recombination

RS constitute DNA sequences where recombinase binding and strand exchange takes place. The disposition of the involved RS in a site-specific recombination reaction plays a crucial role for its outcome and biological function. Intramolecular recombination between direct repeated RS usually results in excision of the interjacent DNA fragment, whereas recombination between inverted repeated RS inverts the fragment flanked by the two RS. Recombination between two RS present on separated DNA molecules leads to integration of one molecule into the other (Hallet and Sherratt, 1997).

Excision events usually resolve multimers of circular replicons (e.g. bacterial chromosomes, plasmids, circular phage genomes), which may arise through homologous recombination events after replication and therefore play an important role during segregation of genetic material to daughter cells. Also excision and integration allow switching between the lytic and lysogenic life cycles of temperate bacteriophages (Barksdale and Arden, 1974; Landy, 1989). In contrast, site-specific inversions are usually associated with the generation of genetic diversity by controlling the expression of phase variable proteins (e.g. expression of surface antigens, flagella proteins, tail fibers of bacteriophages) (Nash, 1996).

Typically, RS of the λ-integrase family contain a central part of 6-8 bp that is referred to as the overlap region within which strand cutting and exchange occurs. The overlap region is flanked by two inverted repeated sequences that act as recombinase binding elements. Besides the overlap region and the recombinase binding elements, both of which constitute the functional core of the RS, many site-specific recombination systems require additional factors (e.g. extra DNA binding sites for accessory proteins, precise DNA structure) that control the outcome of the reaction (Nash, 1996; Hallet and Sherratt, 1997).

3.4.4 *Mycoplasma* site-specific recombinases

Analysis of the amino acid sequences of *Mycoplasma* proteins (Xer1 of *M. agalactiae*, Mbr of *M. bovis* and HvsR of *M. pulmonis*) that are associated with phase variation by site-specific DNA rearrangements revealed that these proteins are members of the λ-integrase family (Ron et al., 2002). The C-terminal regions of these proteins carry the R-H-R-Y tetrad that is involved in catalysis. The C-terminal part of *Mycoplasma* recombinases was also shown to exhibit significant sequence similarities with the XerC and XerD recombinases of *E. coli* (Ron et al., 2002) that resolve multimeric forms of circular replicons to allow efficient segregation of monomers to daughter cells at cell division (Blakely et al., 1991; Blakely et al., 1993).

3.5 Work description

The work presented in this thesis focuses on the dynamics of Vpma surface variation in the ruminant pathogen *M. agalactiae*. The publication '**Xer1 Mediated Site-specific DNA-Inversions and Excisions in *Mycoplamsa agalactiae***' describes recombination experiments carried out in *E. coli*, which clearly demonstrate that the Xer1 recombinase catalyzes *vpma* gene inversions at conserved *vpma* RS located in the 5' UTR of all six *vpma* genes. The orientation of the RS was shown to be crucial for the outcome of the recombination reaction. Recombination between inverted repeated RS led to inversions, whereas direct repeated alignment of the RS resulted in excisions. Furthermore, Xer1-mediated excision events were also successfully demonstrated in *M. agalactiae*, using a newly developed *lacZ* gene-based assay, which is the first application of this reporter system in *M. agalactiae*.

Another ongoing project deals with the evaluation of the biological significance of Vpma proteins and their phase variation in pathogenesis. To adress this question, animal infection trials were performed using *xer1*-deficient PLMs that were previously demonstrated to steadily express a single Vpma product for many generations during in vitro growth (Chopra-Dewasthaly et al., 2008). Interestingly, PLMs isolated from the animals displayed new Vpma phenotypes. The collection of data '**Xer1-independent Mechanisms of Vpma Phase Variation are Triggered by the Presence of Vpma-specific Antibodies**' deals with the molecular characterization of novel escape mechanims that resulted in Vpma switching in these PLM clones in the absence of the Xer1 recombinase. Complex DNA rearrangements including generation of hybrid *vpma* genes, as well as gene duplications and deletions were triggered most likely by the host immune response. Similar mechanisms could be demonstrated in vitro under the immune pressure of the Vpma-specific antibodies. These results cleary demonstrate the requirement of Vpma surface alteration as a survival strategy in the native host and provide important data for studying similar antigenic variation systems in other *Mycoplasma* species.

4 References Introduction

Barksdale, L., and S. B. Arden. 1974. Persisting bacteriophage infections, lysogeny, and phage conversions. Annu. Rev. Microbiol. **28:**265-299.

Bergonier, D., X. Berthelot, and F. Poumarat. 1997. Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. Rev. Sci. Tech. **16:**848-873.

Blakely, G., S. Colloms, G. May, M. Burke, and D. Sherratt. 1991. *Escherichia coli* XerC recombinase is required for chromosomal segregation at cell division. New Biol. **3:**789-798.

Blakely, G., G. May, R. McCulloch, L. K. Arciszewska, M. Burke, S. T. Lovett, and D. J. Sherratt. 1993. Two related recombinases are required for site-specific recombination at *dif* and *cer* in *E. coli* K12. Cell **75:**351-361.

Chopra-Dewasthaly, R., C. Citti, M. D. Glew, M. Zimmermann, R. Rosengarten, and W. Jechlinger. 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. Mol. Microbiol. **67:**1196-1210.

Chopra-Dewasthaly, R., M. Marenda, R. Rosengarten, W. Jechlinger, and C. Citti. 2005. Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. FEMS Microbiol. Lett. **253:**89-94.

Chopra-Dewasthaly, R., M. Zimmermann, R. Rosengarten, and C. Citti. 2005. First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn*4001*mod. Int. J. Med. Microbiol. **294:**447-453.

Citti, C., G. F. Browning, R. Rosengarten. 2005. Phenotypic diversity and cell invasion in host subversion by pathogenic mycoplasmas,, p. 439–484. *In* A. B. a. G. F. Browning (ed.), Mycoplasmas: molecular biology, pathogenicity and strategies for control. Horizon Bioscience, Norfolk, United Kingdom.

Citti, C., and R. Rosengarten. 1997. Mycoplasma genetic variation and its implication for pathogenesis. Wien Klin. Wochenschr. **109:**562-568.

Corrales, J. C., A. Esnal, C. De la Fe, A. Sanchez, P. Assuncao, J. B. Poveda, A. Contreras. 2007. Contagious agalactia in small ruminants. Small Ruminant Research **68:**154–166.

Flitman-Tene, R., S. Mudahi-Orenstein, S. Levisohn, and D. Yogev. 2003. Variable lipoprotein genes of *Mycoplasma agalactiae* are activated in vivo by promoter addition via site-specific DNA inversions. Infect. Immun. **71:**3821-3830.

Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, R. D Fritchman. J. F. Weidman, K. V. Small, M. Sandusky, J. Fuhrmann, D. Nguyen, R. Utterback, D. M. Saudek, C. A. Phillips, J. M. Merrick, J.-F. Tomb, B. A. Dougherty, K. F. Bott, P.-C. Hu, T. S. Lucier, S. N. Peterson, H. O. Smith, C. A. Hutchison III , J. C. Venter. 1995. The minimal gene complement of *Mycoplasma genitalium*. Science **270:** 397-403.

Glew, M. D., M. Marenda, R. Rosengarten, and C. Citti. 2002. Surface diversity in *Mycoplasma agalactiae* is driven by site-specific DNA inversions within the *vpma* multigene locus. J. Bacteriol. **184:**5987-5998.

Glew, M. D., L. Papazisi, F. Poumarat, D. Bergonier, R. Rosengarten, and C. Citti. 2000. Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant variable surface proteins in *Mycoplasma agalactiae*. Infect. Immun. **68:**4539-4548.

Hacker, J., G. Blum-Oehler, I. Mühldorfer, and H. Tschäpe. 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23:**1089-1097.

Hallet, B., and D. J. Sherratt. 1997. Transposition and site-specific recombination: adapting DNA cut-and-paste mechanisms to a variety of genetic rearrangements. FEMS Microbiol. Rev. **21:**157-178.

Horino, A., Y. Sasaki, T. Sasaki, and T. Kenri. 2003. Multiple promoter inversions generate surface antigenic variation in *Mycoplasma penetrans*. J. Bacteriol. **185:**231-142.

Lambert, M. 1987. Contagious agalactia of sheep and goats.*In* Mycoplasmoses of ruminants. Rev. Sci. Tech. OIE. **6**: 699-711

Landy, A. 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annu. Rev. Biochem. **58:**913-949.

Madanat, A., Z. Zendulkova, Z. Pospisil. 2001. Contagious Agalactia of Sheep and Goats. A Review. ACTA VET. BRNO 2001 **70:**403-412.

Manso-Silvan, L., E. M. Vilei, K. Sachse, S. P. Djordjevic, F. Thiaucourt, J. Frey. 2009. *Mycoplasma leachii* sp. nov. as a new species designation for *Mycoplasma* sp. bovine group 7 of Leach, and reclassification of *Mycoplasma mycoides* subsp. *mycoides* LC as serovar of *Mycoplasma mycoides* subsp. *capri*. Int. J. Syst. Evol. Microbiol. **59**: 1353-1358.

McGarrity, G. J., H. Kotani, G. H. Butler. 1992. Mycoplasmas and tissue culture cells, p. 445–454. *In* J. Manillof, Mcelhaney, N., Finch, L.R., Baseman, J.B. (ed.), Mycoplasmas, Molecular Biology and Pathogenesis. American Society for Microbiology, Washington, USA.

Nash, H. A. 1996. Site-Specific Recombination: Integration, Excision, Resolution, and Inversion of Defined DNA Segments, p. 2363-2376. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology. ASM Press, Washington, D.C.

Neyrolles, O., I. Chambaud, S. Ferris, M. C. Prevost, T. Sasaki, L. Montagnier, and A. Blanchard. 1999. Phase variations of the *Mycoplasma penetrans* main surface lipoprotein increase antigenic diversity. Infect. Immun. **67:**1569-1578.

Nicholas, R. A., R. D. Ayling, and L. McAuliffe. 2009. Vaccines for *Mycoplasma* diseases in animals and man. J. Comp. Pathol. **140:**85-96.

Nicholas, R. A. J. 2002. Improvements in the diagnosis and control of diseases of small ruminants caused by mycoplasmas. Small Ruminant Research **45:**145–149.

Nouvel, L. X., M. Marenda, P. Sirand-Pugnet, E. Sagne, M. Glew, S. Mangenot, V. Barbe, A. Barre, S. Claverol, and C. Citti. 2009. Occurrence, plasticity, and evolution of the *vpma* gene family, a genetic system devoted to high-frequency surface variation in *Mycoplasma agalactiae*. J. Bacteriol. **191:**4111-4121.

Nunes-Düby, S. E., H. J. Kwon, R. S. Tirumalai, T. Ellenberger, and A. Landy. 1998. Similarities and differences among 105 members of the Int family of site-specific recombinases. Nucleic Acids Res. **26:**391-406.

Osawa, S., T. H. Jukes, K. Watanabe, and A. Muto. 1992. Recent evidence for evolution of the genetic code. Microbiol. Rev. **56:**229-264.

Rawadi, G., and O. Dussurget. 1995. Advances in PCR-based detection of mycoplasmas contaminating cell cultures. PCR Methods. Appl. **4:**199-208.

Razin, S. 1999. Adherence of pathogenic mycoplasmas to host cells. Biosci. Rep. **19:**367-372.

Razin, S. 1997. Comparative genomics of mycoplasmas. Wien Klin. Wochenschr. **109:**551-556.

Razin, S., D. Yogev, and Y. Naot. 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiol. Mol. Biol. Rev. **62:**1094-1156.

Ron, Y., R. Flitman-Tene, K. Dybvig, and D. Yogev. 2002. Identification and characterization of a site-specific tyrosine recombinase within the variable loci of *Mycoplasma bovis*, *Mycoplasma pulmonis* and *Mycoplasma agalactiae*. Gene **292:**205-211.

Rosengarten, R., C. Citti, M. Glew, A. Lischewski, M. Droesse, P. Much, F. Winner, M. Brank, J. Spergser. 2000. Host-pathogen interactions in mycoplasma pathogenesis: virulence and survival strategies of minimalist prokaryotes. Int. J. Med. Microbiol. **290**: 15-35

Rosengarten, R., and K. S. Wise. 1990. Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. Science **247:**315-318.

Rosengarten, R., and K. S. Wise. 1991. The Vlp system of *Mycoplasma hyorhinis*: combinatorial expression of distinct size variant lipoproteins generating high-frequency surface antigenic variation. J. Bacteriol. **173:**4782-4793.

Röske, K., A. Blanchard, I. Chambaud, C. Citti, J. H. Helbig, M. C. Prevost, R. Rosengarten, and E. Jacobs. 2001. Phase variation among major surface antigens of *Mycoplasma penetrans*. Infect. Immun. **69:**7642-7651.

Rottem, S. and Naot, Y. 1998. Subversion and exploitation of host cells by mycoplasmas. Trends Microbiol. **6**: 436-440.

Sasaki, Y., J. Ishikawa, A. Yamashita, K. Oshima, T. Kenri, K. Furuya, C. Yoshino, A. Horino, T. Shiba, T. Sasaki, and M. Hattori. 2002. The complete genomic sequence of *Mycoplasma penetrans*, an intracellular bacterial pathogen in humans. Nucleic Acids Res. **30:**5293-5300.

Sirand-Pugnet, P., C. Lartigue, M. Marenda, D. Jacob, A. Barre, V. Barbe, C. Schenowitz, S. Mangenot, A. Couloux, B. Segurens, A. de Daruvar, A. Blanchard, and C. Citti. 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet. **3:**744-758.

Sitaraman, R., A. M. Denison, and K. Dybvig. 2002. A unique, bifunctional site-specific DNA recombinase from *Mycoplasma pulmonis*. Mol. Microbiol. **46:**1033-1040.

Smith, M. C., and H. M. Thorpe. 2002. Diversity in the serine recombinases. Mol. Microbiol. **44:**299-307.

van der Woude, M. W., and A. J. Baumler. 2004. Phase and antigenic variation in bacteria. Clin. Microbiol. Rev. **17:**581-611

Wise, K. S. 1993. Adaptive surface variation in mycoplasmas. Trends Microbiol. **1:**59–63.

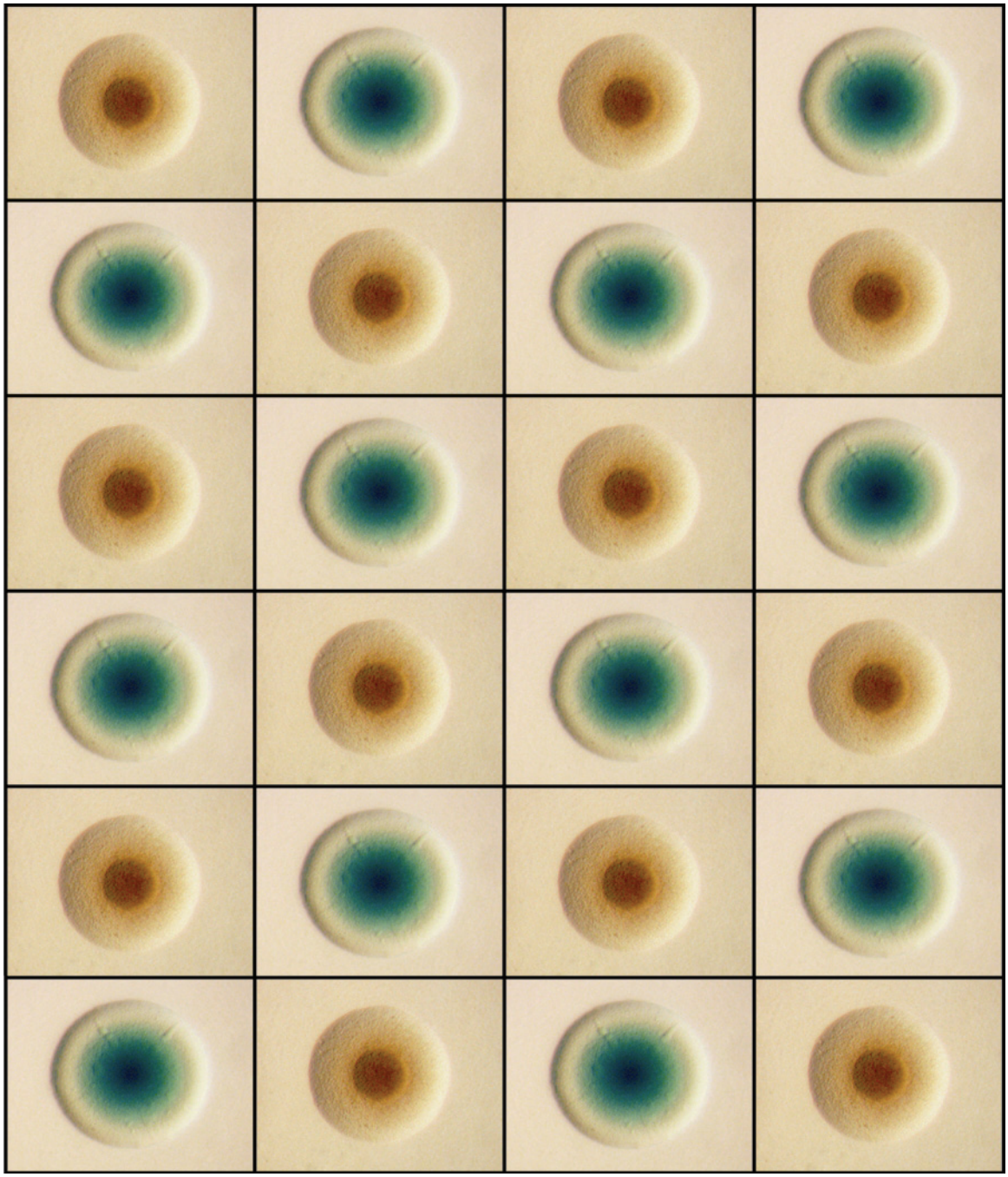
Woese, C. R., J. Maniloff, and L. B. Zablen. 1980. Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. U S A **77:**494-498.

Yogev, D., R. Rosengarten, R. Watson-McKown, and K. S. Wise. 1991. Molecular basis of Mycoplasma surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5' regulatory sequences. Embo J. **10:**4069-4079.

5 Results and Publications

5.1 Publication: Xer1-Mediated Site-Specific DNA Inversions and Excisions in *Mycoplasma agalactiae*

COVER ILLUSTRATION:



JB Accepts, published online ahead of print on 18 June 2010
J. Bacteriol. doi:10.1128/JB.01537-09

Xer1-Mediated Site-Specific DNA Inversions and Excisions in *Mycoplasma agalactiae*

Stefan Czurda, Wolfgang Jechlinger[†],

Renate Rosengarten, and Rohini Chopra-Dewasthaly[*]

Institute of Bacteriology, Mycology and Hygiene (IBMH), Department of Pathobiology,

University of Veterinary Medicine Vienna, Veterinaerplatz 1, A-1210 Vienna, Austria

[*]Corresponding author. Phone: +43-1-25077-2101. Fax: +43-1-25077-2190.

E-mail: Rohini.Chopra-Dewasthaly@vetmeduni.ac.at

[†]Deceased

Running Title: Xer1-Mediated Recombination in *Mycoplasma agalactiae*

Abstract

Surface antigen variation in *Mycoplasma agalactiae,* the etiological agent of Contagious Agalactia in sheep and goats, is governed by site-specific recombination within the *vpma* multigene locus encoding the Vpma family of variable surface lipoproteins. This high-frequency Vpma phase switching was previously shown to be mediated by a Xer1 recombinase encoded adjacent to the *vpma* locus. In this study it was demonstrated in *E. coli* that the Xer1 recombinase is responsible for catalyzing *vpma* gene inversions between recombination sites (RS) located in the 5' untranslated region (UTR) of all six *vpma* genes causing cleavage and strand exchange within a 21 bp conserved region that serves as a recognition sequence. It was further shown that the outcome of the site-specific recombination event depends on the orientation of the two *vpma* RS as direct or inverted repeats. While recombination between inverted *vpma* RS led to inversions, recombination between direct repeated *vpma* RS led to excisions. Using a newly developed excision assay based on the *lacZ* reporter system, we were able to successfully demonstrate under native conditions that such Xer1-mediated excisions can indeed also occur in *M. agalactiae* type strain PG2, whereas they were not observed in the control *xer1*-disrupted VpmaY phase-locked mutant (PLMY) which lacks Xer1 recombinase. Unless there are specific regulatory mechanisms preventing such excisions, this might be the cost that the pathogen has to render at a population level for maintaining this high-frequency phase variation machinery.

INTRODUCTION

Members of the bacterial class *Mollicutes,* which are generally referred to as mycoplasmas are considered one of the simplest self-replicating prokaryotes carrying minimal genomes. Even though having lost many biosynthetic pathways during a reductive evolution, mycoplasmas represent important pathogens of humans, animals and plants, as they are equipped with sophisticated molecular mechanisms allowing them to spontaneously change their cell-surface repertoire to persist in immune-competent hosts (25).

The important ruminant pathogen *Mycoplasma agalactiae* causes Contagious Agalactia in sheep and goats and exhibits antigenic diversity by site-specific DNA rearrangements within a pathogenicity island-like gene locus (9, 10, 26). The so-called *vpma* locus constitutes a family of six distinct but

related genes that encode major immunodominant membrane lipoproteins, the Vpmas (variable proteins of *Mycoplasma agalactiae*) (10, 11). These surface-associated proteins vary in expression at an unusually high frequency, and only one *vpma* gene is transcribed at a time from a single promoter present in that locus, while all other genes are silent (9, 10). An ORF having homology to the λ-integrase family of site-specific recombinases was found in the vicinity of the *vpma* locus and was predicted to mediate DNA inversions responsible for switching the promoter from an active *vpma* gene to a silent one, resulting in alteration of *vpma* expression (9, 10). This recombinase, designated as Xer1, was recently demonstrated to be indeed responsible for phase variation of Vpma proteins (4). Targeted knockouts of the *xer1* gene by homologous recombination prevented Vpma switching and produced Vpma phase-locked mutants (PLMs) steadily expressing a single *vpma* gene without any variation. Complementation of the wild-type *xer1* gene in these PLMs restored Vpma phase variation (4). Similar systems generating surface diversity by DNA inversions involving site-specific recombination have been identified in other mycoplasma species (3, 18, 26).

Site-specific recombination systems are widespread among bacteria, and the biological functions of these systems strongly depend on the participating recombination sites (RS) (16, 24, 27). Excision events between direct repeated RS usually resolve chromosome- or plasmid-dimers, which can arise through homologous recombination, ensuring proper segregation of newly replicated genetic material to daughter cells (1). Also, site-specific recombination mediates integration and excision of phage genomes into and out of the host chromosome (13). In contrast, site-specific inversion involving inverted repeated RS generates genetic diversity and often controls the expression of genes that are important for pathogenesis (21).

The Xer1 recombinase of *M. agalactiae* belongs to the λ-integrase family of site-specific recombinases (10). Members of this family share four strongly conserved amino acid residues (R-H-R-Y) within the C-terminal half of the protein. This tetrad includes the active tyrosine residue that is directly involved in the recombination reaction (8). Recombination occurs by formation and resolution of a Holliday junction intermediate involving a covalent linkage between the recombinase and the DNA through the tyrosine residue. Since energy co-factors such as ATP are not required, such recombination events can occur in the absence of replication (16, 24).

Sequence alignment of *vpma* genes identified a conserved 21-bp region within the 5' untranslated region (UTR) of all *vpma* genes that was predicted to be involved in Xer1-mediated inversions (10). The present study clearly demonstrates that the Xer1 recombinase recognizes RS located within the 5' UTR of *vpma* genes causing cleavage and strand exchange within a conserved region of 21 bp. By placing two *vpma*-derived RS on a plasmid along with the *xer1* gene, recombination events were demonstrated in *E. coli* upon Xer1 induction via PCR and restriction analysis. Although the conserved 21-bp region was sufficient for inversions, additional nucleotides flanking it at the 5' end were found to have a positive influence on the rate of recombination. An interesting outcome of these studies was that Xer1 also mediates excisions between direct repeated *vpma*-RS in *E.coli*. This raised the intriguing possibility that such Xer1-mediated excisions also occur in the native *M. agalactiae* system. For further analysis of such excision events in the native system, we tested the feasibility of using the *lacZ* reporter tool in *M. agalactiae,* as *lacZ* is known to be successfully expressed in few other mycoplasma species to study gene expression by promoter probe vectors (15, 19, 22, 23). We developed an excision assay based on blue/white phenotype selection to study Xer1-mediated excisions in *M. agalactiae*, thus displaying a novel application of the *lacZ* reporter gene in mycoplasmas. Successful implementation of this reporter system demonstrated Xer1-mediated excisions in the *M. agalactiae* type strain PG2 based on blue/white selection and PCR analysis. As expected, such excisions were not observed in the control *xer1*-disrupted VpmaY phase-locked mutant (PLMY) which lacks Xer1. Excisions in the native system imply that genetic material is susceptible to loss, which might be the cost for maintaining the machinery of high-frequency gene shuffling for a greater population advantage, unless there are specific regulatory mechanisms preventing such excisions.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. *E. coli* strain DH10B (Invitrogen GmbH, Lofer, Austria) was used for cloning and expression of the *xer1* gene. Plasmids used for Xer1-mediated excision and inversion experiments in *E. coli* were derived from pBAD24 (14), and plasmid constructs for studying excisions in *M. agalactiae* were derived from pISM2062 (22). Plasmids p5H1.8 (11), p5H4.7 (10) and pAWC10-lac (20) have been described elsewhere. Excision studies were carried out

within *M. agalactiae* type strain PG2 (32) and the *xer1*-disruptant strain PLMY (4). Cells were grown in SP4 (33) broth and transformants were selected on SP4 agar plates containing gentamicin (50 µg/ml) and/or tetracycline (2 µg/ml) as appropriate. To monitor *lacZ* expression on the basis of blue/white selection, *M. agalactiae* cells carrying the *lacZ* gene were grown on SP4 agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at a concentration of 160 µg/ml.

DNA manipulations. Preparation of plasmid and genomic DNA and isolation of DNA fragments from agarose gels were carried out using suitable kits from Promega (Promega Wizard SV Gel and PCR Clean-Up System, Promega, Mannheim, Germany), PEQLAB (EZNA Plasmid Miniprep Kit I, PEQLAB-Biotechnologie GmbH, Erlangen, Germany) and QIAGEN (QIAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany). Restriction endonucleases and nucleotides were purchased from Promega, T4 DNA Ligase from Roche (Roche Diagnostics, Vienna, Austria) and Antarctic Phosphatase from New England Biolabs (New England Biolabs, Frankfurt am Main, Germany). Transformation of *E. coli* cells was performed by electroporation with a BIO-RAD Gene-Pulser^{R}II (Bio-Rad Laboratories GmbH, Vienna, Austria) using 1.25 V voltage, 25 µF capacitance and 200 Ω resistance. Transformation of *M. agalactiae* cells was carried out as described (5). Oligonucleotide synthesis and sequencing were carried out at VBC-Biotech Service (VBC-Biotech Service GmbH, Vienna, Austria). Standard molecular procedures were performed as described by Sambrook et al (28).

***xer1* induction experiments in *E. coli*.** *E. coli* DH10B transformed with plasmids used for inversion and excision experiments were plated on LB agar plates containing ampicillin (100 µg/ml) and 5% glucose for inhibition of basal expression from the P_{BAD} promoter and grown overnight at 28°C. Individual transformants were picked, inoculated in LB broth containing ampicillin and glucose and grown overnight at 28°C. For *xer1* induction, 600 µl of the overnight culture was transferred into 60 ml LB broth containing 100 µg/ml ampicillin and cells were grown at 37°C. Growth of bacteria was monitored by measurement of the optical density at 600 nm (OD_{600}). At an OD_{600} of 0.3, *xer1* expression was induced by adding 0.5% L-arabinose. Samples for plasmid preparations were removed

at different time points (2 h, 4 h, 6 h and 20 h) as appropriate. Samples for plasmid preparation of uninduced cultures serving as negative controls were taken from overnight cultures and/or at the beginning of induction. Plasmid samples were used for subsequent PCR- and restriction analysis assays.

PCR amplification. PCR reactions for cloning (except amplification of the *lacZ* gene) were carried out in a total volume of 100 µl consisting of 50 ng template DNA, 1 µM of each primer, 25 mM $MgCl_2$, 0.2 µM dNTPs and 5 U of Taq DNA polymerase (Promega) in 1 x PCR buffer supplied by the manufacturer. Cycling parameters consisted of 1 cycle of 3 min at 94°C for initial denaturation followed by 30 cycles of 1 min denaturation at 94°C, 1 min at various annealing temperatures (60°C for *xer1*, 58-63°C for the 200-bp *vpmaY*-RS, 57°C for RS111Y and 58°C for RS184U) and 1 min at 72°C for extension, and a final extension step of 5 min at 72°C.

The *lacZ* gene with its native Shine-Dalgarno sequence from *E. coli* was amplified in a 100 µl mix constituting 50 ng of plasmid pAWC10-lac, 1 µM of primers LacfwBN (containing *Bam*HI and *Nco*I sites for subsequent clonig) and LacrvSB (containing *Sma*I and *Bgl*II sites), 0.2 µM dNTPs, 1 U of Long PCR Enzyme mix (Fermentas GmbH, St. Leon-Rot, Germany) and 1 x PCR buffer (containing 25mM $MgCl_2$) supplied by the manufacturer. Cycling conditions consisted of 1 cycle of 2 min at 94°C for initial denaturation, 10 cycles of 20 s at 94°C for denaturation and 3 min at 68°C for extension, followed by 20 cycles of 20 s at 94°C for denaturation and 3 min 20 s at 68°C for extension and a final extension step of 10 min at 68°C.

A three primer hot-start PCR for detection of inversion events was accomplished in a 25 µl volume containing 25 ng of template DNA, 1 µM of primers P1 and P2, 0.2 µM of primer P4, 2.5 mM $MgCl_2$, 0.2 µM dNTPs, 1 M betaine and 5 U of HOT FIREPol DNA polymerase I (Solis BioDyne OU, Tartu, Estonia) in 1 x PCR buffer supplied by the manufacturer. Cycling parameters consisted of 1 cycle of 15 min at 95°C for initial denaturation, followed by 30 cycles of 43 s denaturation at 94°C, 43 s annealing at 61°C and 1 min 33 s at 72°C for extension and a final extension step of 10 min at 72°C.

PCR for the detection of Xer1-mediated excision events in *M. agalactiae* was carried out in a volume of 25µl, which consisted of 10 ng genomic DNA obtained from PG2 or PLMY transformants, 1 µM of

primers 184Ubfw and ISR-f, 2.5 mM $MgCl_2$, 0.2 µM dNTPs and 5 U of HOT FIREPol DNA polymerase I (Solis BioDyne OU, Tartu, Estonia) in 1 x PCR buffer supplied by the manufacturer. Cycling parameters consisted of 1 cycle of 15 min at 95°C for initial denaturation, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 3 min 50 s extension at 72°C and a final extension step of 5 min at 72°C.

Plasmid constructions. *Construction of plasmids pBADRS21x2 and pBADXerRS21x2.* 2 µg of each of the primers Ol1new2sen and Ol1new2asen (oligonucleotides are summarized in Table 1) were mixed with 46 µl of annealing buffer (100 mM potassium acetate, 30 mM HEPES, 2 mM magnesium acetate, pH 7.4), boiled at 100°C for 5 min and cooled down gradually to room temperature within 60 min. The resulting double-stranded 21-bp RS contained *Hind*III and *Xba*I overhangs and was ligated into *Hind*III and *Xba*I sites of pBAD24. The ligation mixture was transformed into *E. coli* DH10B by electroporation as described resulting in plasmid pBADRS21. Another 21-bp RS was introduced into the *Nsi*I and *Nar*I sites of pBADRS21 to obtain a vector with two inverted repeated 21-bp RS. The second 21-bp RS containing *Nsi*I and *Nar*I overhangs was similarly obtained by annealing oligonucleotides RS21NsiI and RS21NarI and ligated into the corresponding sites of pBADRS21 to obtain vector pBADRS21x2 containing two inverted repeats of the 21-bp RS. The *xer1* gene was amplified from plasmid p5H4.7 using primers RecATGET28 (containing *Nco*I site) and XerrvSma (containing *Sma*I site). The 750-bp product was digested with *Nco*I and *Sma*I and cloned at the corresponding sites of pBADRS21x2 resulting in plasmid pBADXerRS21x2.

Construction of plasmids pBADXerIR200Y and pBADXerDR200Y. A 200-bp fragment containing the *vpma*Y-RS corresponding to the 5' UTR of *vpmaY* gene was obtained by PCR amplification as described using plasmid p5H1.8 as template and oligonucleotides RSmcsX (containing a *Xba*I site) and RSmcsH (containing *Hind*III site) as primers. The *Hind*III- and *Xba*I-digested amplicon was then ligated into the corresponding sites of pBAD24 resulting in plasmid pBADRS. Another copy of the 200-bp fragment was introduced into plasmid pBADRS both as a direct and as an inverted repeat after PCR amplification from plasmid p5H1.8 using oligonucleotide pair Rsd1cla (containing a *Cla*I site) and Rsd1nsi (containing a *Nsi*I site) for direct repeated RS, and oligonucleotide pair Rsd2cla

(containing a *Cla*I site) and Rsd2nsi (containing a *Nsi*I site) for inverted repeated RS. The 200-bp PCR fragments were cut with *Cla*I and *Nsi*I and introduced into *Nsi*I and *Nar*I cut plasmid pBADRS. *Nar*I and *Cla*I produce compatible cohesive ends and therefore *Nar*I overhangs can be ligated to *Cla*I overhangs. The two plasmids were designated pBADDR (direct repeated RS) and pBADIR (inverted repeated RS). The *xer1* gene was introduced into plasmids pBADDR and pBADIR as described for plasmid *pBADXerRS21x2* resulting in plasmids carrying two 200-bp RS either as inverted (pBADXerIR200Y) or as direct repeats (pBADXerDR200Y) along with the *xer1* gene.

Construction of plasmids pBADXerIR21Y, pBADXerIR31Y3', pBADXerIR56Y3', pBADXerIR57Y5' and pBADXerIR111Y. Oligonucleotides Ol1new2sen and Ol1new2asen were annealed to obtain RS21Y, Oligo#2sense and Oligo#2asense for RS31Y3', Oligo#3sense and Oligo#3asense for RS56Y3' and Ol9sen and Ol9asen for RS57Y5'. The annealed oligonucleotides carried *Xba*I and *Hind*III overhangs for cloning into the corresponding sites of pBADXerIR200Y resulting in plasmids pBADXerIR21Y, pBADXerIR31Y3', pBADXerIR56Y3' and pBADXerIR57Y5', respectively. RS111Y was obtained by PCR as described using plasmid p5H1.8 as template and oligonucleotides pBADoligo#4fw (containing a *Xba*I site) and pBADoligo#4rv (containing a *Hind*III site) as primers. The *Hind*III- and *Xba*I-cut PCR product was introduced into the corresponding sites of pBADXerIR200Y resulting in plasmid pBADXerIR111Y.

Construction of plasmids pBADXer200Y/184U, pBADXerIR200Y/56X5' and pBADXer53U5'/57Y5'. Plasmid pBADXer200Y/184U was obtained by replacing the *Xba*I- and *Hind*III-cloned 200-bp *vpmaY* RS of pBADXerIR200Y with a 184-bp *vpmaU*-RS generated by PCR using plasmid p5H4.7 as template and PrimerRSU (containing a *Xba*I site) and RSmcsH (containing a *Hind*III site) as primers. The PCR fragment restricted with *Hind*III and *Xba*I was cloned into the corresponding sites of vector pBADXerIR200Y resulting in plasmid pBADXerIR200Y/184U containing a 200-bp *vpmaY*-RS and a 184-bp *vpmaU*-RS aligned as inverted repeats. Similarly plasmid pBADXerIR200Y/56X5' was obtained by replacing the *Xba*I- and *Hind*III-cloned 200-bp *vpmaY* RS with a 56-bp RS originating from the *vpmaX* gene. Oligonucleotides OligoRSXsen and OligoRSXasen were annealed as described. The obtained fragment containing *Xba*I and *Hind*III overhangs was cloned into the corresponding sites of vector pBADXerIR200Y resulting in plasmid pBADXerIR200Y/56X5' containing a 200-bp

vpmaY-RS and a 56-bp *vpmaX*-RS as inverted repeats. Plasmid pBADXer53U5'/57Y5' was obtained by replacing the *Nar*I/*Cla*I and *Nsi*I cloned 200-bp *vpmaY* RS of pBADXerIR57Y5' with a 53-bp *vpmaU*-RS generated by annealing oligonucleotides OligoRSUsen and OligoRSUasen as described. The obtained fragment contained a *Msc*I blunt end and a *Bst*API overhang for cloning at the respective sites in plasmid pBADXerIR57Y5' resulting in plasmid pBADXer53U5'/57Y5' containing a 57-bp *vpmaY*-RS and a 53-bp *vpmaU*-RS as inverted repeats.

Construction of plasmids pIL and pILDR. The *lacZ* gene with its native Shine-Dalgarno sequence from *E. coli* was obtained by PCR as described (see section PCR amplication). The *Bam*HI- and *Sma*I-cut 3.1 kb product was introduced into the corresponding sites present in the left IS element of transposon Tn*4001*mod in plasmid pISM2062 resulting in plasmid pIL. The 184-bp *vpma*U-RS was amplified as described for the construction of pBADXer200Y/184U except that oligonucleotides 184Ubfw (containing a *Bam*HI site) and 184Unrv (containing a *Nco*I site) were used as primers. The *Bam*HI- and *Nco*I-cut product was introduced into the corresponding sites of pIL resulting in vector pILRSU carrying the *lacZ* gene flanked by a 184-bp *vpma*U RS at the 5' end. The 200-bp *vpma*Y RS was amplified as described for construction of pBADXer200Y except that oligonucleotides 200YBfw and 200YBrv (both containing *Bgl*II sites) were used as primers. The PCR fragment restricted with *Bgl*II, was introduced into the *Bgl*II site of dephosphorylated plasmid pILRSU. The direct repeated orientation of this fragment was verified by sequencing. The resulting vector pILDR contained the *lacZ* gene flanked by a 184-bp *vpma*U-RS at the 5' end and a 200-bp *vpma*Y-RS at the 3' end aligned as direct repeats.

Restriction analysis and agarose gel electrophoresis of recombination products. Recombination products derived from plasmid pBADXerDR200Y containing direct repeated RS were applied directly on a 1% (w/v) agarose gel or after linearization with *Xba*I and *Hind*III restriction endonucleases. Inversion events within plasmids containing two inverted repeated RS (pBADRS21x2, pBADXerRS21x2, pBADXerIR200Y pBADXerIR21Y, pBADXerIR31Y3', pBADXerIR56Y3', pBADXerIR57Y5', pBADXerIR111Y, pBADXer200Y/184U, pBADXerIR200Y/56X5' and

pBADXer53U5'/57Y5') were verified by applying the *Hind*III- and *Eco*RV-digested samples on a 1% (w/v) agarose gel and visualizing under UV light after ethidium bromide staining.

RESULTS

A conserved 21-bp sequence common to all *vpma* genes is sufficient for Xer1-mediated inversion. Alignment of the 5' UTR region of all six *vpma* genes present in clone 55-5 of type strain PG2 identified a conserved 21-bp region (TTGATATTTATTAATAGATTT) thought to be involved in Xer1-mediated recombination (10). To verify if this 21-bp RS is indeed involved and is sufficient for recombination, two inverted repeated 21-bp RS were introduced into plasmid pBAD24 together with the *xer1* gene, which was placed under the transcriptional control of the arabinose promoter. The resulting plasmid pBADXerRS21x2 was introduced into *E. coli,* and recombination events were followed by testing the plasmid preparations obtained from samples removed after 6 and 20 h of *xer1* induction. Uninduced samples of overnight grown cells carrying pBADXerRS21x2 and cells carrying pBADRS21x2 lacking the *xer1* gene served as negative controls. For detection of inversion products, first a restriction analysis method was used, whereby the plasmid DNA was digested using *Hind*III and *Eco*RV restriction endonucleases and observed for appearance of recombination products upon agarose gel electrophoresis. An inversion event between the two inverted repeated RS in pBADXerRS21x2 would place the *Hind*III site near the *Eco*RV site (Fig. 1A) and would result in the appearance of restriction fragments of 4.7 kb and 0.4 kb beside the two original non-recombined plasmid fragments of 3.4 kb and 1.7 kb if sufficient amount of recombined product is present after *xer1* induction. However, using this restriction analysis method we could not detect any inversion products for both samples taken 6 and 20 h after *xer1* induction (data not shown). To check if the inversion events between two 21-bp RS occur at levels much below the detection limit of the restriction analysis assay, a more sensitive three-primer PCR assay was developed (Fig. 1). Primer P1 (anneals to a region of the *araC* gene) and primer P2 (anneals to a region of the *bla* gene) enable the amplification of a 1 kb 'recombinant' fragment only in case of an inversion between the two RS (Fig. 1A), whereas primer P1 and primer P4 (anneals to a region between the pBR322 *ori* and the *bla* gene) enable the amplification of a 1.4 kb 'non-recombinant' product in the non-recombined vector serving

as an internal positive control in the PCR assay (Fig. 1A and 1B). Beside the 1.4 kb P1-P4 product, samples of pBADXerRS21x2 showed a 1 kb P1-P2 amplicon after 6- and 20-h of *xer1* induction (Fig. 1C, lanes 7 and 8) demonstrating that inversion has occurred between the two inverted repeated 21-bp RS. This 1 kb P1-P2 'recombinant' amplicon was absent in the uninduced sample of pBADXerRS21x2 (Fig. 1C, lane 6) and in samples corresponding to the plasmid pBADRS21x2 (Fig. 1C, lanes 3-5) that carries two inverted repeated 21-bp RS but lacks the *xer1* gene, clearly demonstrating the role of Xer1 recombinase in mediating inversions at the 21-bp RS. As expected, these samples showed amplification of the 1.4 kb P1-P4 'non-recombinant' control product. Also, no recombination was observed in a similar PCR experiment with a control plasmid construct where only one of the two 21-bp RS was present along with the *xer1* gene (data not shown). Furthermore, the 1 kb P1-P2 'recombinant' PCR product obtained with pBADXerRS21x2 when sequenced from both sides generated a sequence that correlates very well to the inversion having occurred at the 21-bp region, because the sequence corresponding to the *Hind*III site was evident in this 1 kb PCR product just next to the RS21 region when sequenced with the P2 primer, which is plausible only if the inversion has occurred in the 21-bp region. Hence, the above PCR and sequencing data are very consistent and indicate that the 21-bp region is sufficient for Xer1-mediated recombination and that this conserved region of the 5' UTR of all *vpma* genes is most likely the site of cleavage and strand-exchange occurring during *vpma* gene inversions.

***vpma* sequences flanking the conserved 21-bp RS enhance the amount of recombinant products to allow detection via restriction analysis assay.** To assess if a larger RS would have an effect and improve the amounts of the inversion products such that they are visible during the restriction analysis assay described earlier, we constructed the plasmid pBADXerIR200Y (Fig. 2A). Two inverted repeated 200-bp RS, each constituting 152 bp of the 5' UTR of the *vpmaY* gene including the conserved 21-bp region along with 48 bp of the coding sequence of *vpmaY* (Fig. 2C), were introduced into plasmid pBAD24 together with the *xer1* recombinase gene. As described before, the inversions were studied after transformation into *E. coli* and upon *xer1* induction. Uninduced samples of overnight grown cultures and samples taken before *xer1* induction served as negative controls.

Induction of *xer1* would result in inversion of the DNA fragment between the two RS, thereby placing the *Hind*III site near the *Eco*RV site (Fig. 2A). Restriction analysis (*Hind*III/*Eco*RV) of the samples removed after 2, 4 and 6 h of Xer1 induction clearly showed a 4.9 kb inversion fragment beside the two original non-recombined plasmid fragments of 3.6 kb and 1.9 kb (Fig. 2B, lanes 4-6). A similar inversion fragment could not be detected when the same amount of plasmid pBADXerRS21x2 containing two 21-bp RS was used in a restriction assay alongside pBADXerIR200Y DNA (data not shown), thereby proving that additional sequences flanking the 21-bp conserved region are instrumental in improving the amounts of Xer1-mediated recombinant products. The second *Hind*III/*Eco*RV inversion fragment of pBADXerIR200Y, which is about 0.6 kb is not visible on the agarose gel in Fig. 2B (lanes 4-6) due to the cumulative effect of its small size and due to small amounts of recombination products present in the total plasmid preparation. The 4.9 kb *Hind*III/*Eco*RV inversion fragment is absent in the uninduced samples (Fig. 2B, lanes 2 and 3), whereas the intensity of this fragment shows a proportional increase with Xer1 induction starting from 2 to 6 h (Fig. 2B, lanes 4-6). It was further confirmed by sequence analysis that the 4.9 kb *Hind*III/*Eco*RV fragment is indeed a product of an inversion event between the two 200-bp RS. These results indicate that sequences in the vicinity of the 21-bp RS improve the amounts of Xer1-mediated inversion products and lead to their detection in the restriction analysis assay, which was not possible with 21-bp RS without flanking sequences.

The Xer1 recombinase mediates excision between direct repeated RS in *E. coli*. The outcome of a recombination event is determined by the alignment of the corresponding RS on a DNA segment (16). Inverted repeated RS enable inversion, whereas direct repeated RS result in excision of the interjacent DNA fragment (16). To analyze the effect of Xer1 recombinase on direct repeated RS in *E. coli*, plasmid pBADXerDR200Y was constructed (Fig. 3A). The *xer1* gene was cloned alongside two 200-bp RS (as depicted in Fig. 2C) identical to those used for inversion studies but this time aligned as direct repeats. Upon *xer1* induction, the DNA sequence flanked by two direct repeated RS was excised and resulted in two excision products: a 3.2 kb replicative miniplasmid (mp) carrying the origin of replication besides the *bla* gene, and a non-replicative 2.3 kb minicircle (mc) carrying the residual

sequence (Fig. 3A). As seen in Figure 3B (lanes 4-7), the supercoiled (sc) forms of the two recombination products (sc mp and sc mc) are visible after 2, 4 and 6 h of *xer1* induction along with the original unrecombined plasmid (sc op). The samples were linearized using *Xba*I and *Hind*III restriction enzymes to further confirm the sizes of the various plasmids. The 5.5 kb original unrecombined plasmid is visible in all samples, both uninduced and induced, whereas the excision products corresponding to the 3.2 kb miniplasmid and 2.3 kb minicircle are visible only upon *xer1* induction (Fig. 3C, lanes 4-7).

Sequences flanking the conserved 21-bp region at the 5' end are required for higher amounts of recombination products. Our results indicate that the 21-bp region, identical in the 5' UTR of all six *vpma* genes, is sufficient for recombination, although a sensitive PCR-based assay is required to verify such site-specific recombinations. On the other hand, inversion between two 200-bp RS including the 21-bp region and additional flanking nucleotides allowed visualization of recombination via the restriction analysis method, indicating that a larger RS favours higher amounts of recombination products. In order to shorten this 200-bp RS and to know if 5' or/and 3' flanking regions of the conserved 21-bp region are critical for the detection of sufficient amounts of recombination products, the restriction analysis method was performed using newly constructed plasmids (pBADXerIR21Y, pBADXerIR31Y3', pBADXerIR56Y3', pBADXerIR57Y5' and pBADXerIR111Y), where one of the two 200-bp RS of plasmid pBADXerIR200Y was successively replaced by shorter versions of different lengths and different flanking regions derived from the *vpmaY* gene as depicted in Fig. 4A. Inversion products between the 200-bp RS and the 21-bp RS in pBADXerIR21Y could not be detected in the restriction analysis method even after 20 h of *xer1* induction (Fig. 4B, lane 4), which confirms our previous results using pBADXerRS21x2 (carrying two 21-bp RS) and acts as a negative control while comparing it with other constructs. Also, extension of the 21-bp region by 10 nucleotides in pBADXerIR31Y3' (Fig. 4A, RS31Y3') and 35 nucleotides in pBADXerIR56Y3' (Fig. 4A, RS56Y3') at the 3' end did not yield any recombination products as seen in Fig. 4B, lanes 5 and 6, respectively. However, addition of 54 nucleotides at the 3' end and 36 nucleotides at the 5' end of the 21-bp region in pBADXerIR111Y (Fig. 4A, RS 111Y) allowed detection of recombination products after restriction

analysis (Fig. 4B, lane 7). Having compared this positive result with that of pBADXerIR56Y3' (Fig. 4A, RS56Y3') which was negative for recombination, one of the obvious difference between the two constructs was the presence of additional 36 nucleotides flanking the 21-bp region at the 5' end. Based on these results, we constructed pBADXerIR57Y5' (Fig. 4A, RS57Y5'), where the 57-bp RS consisted of 21-bp conserved region flanked by additional 36 bp at the 5' end. Indeed, when analyzed by the restriction assay, pBADXerIR57Y5' exhibited detectable amounts of inversion products (Fig. 4B, lane 8). Taken together, these data clearly illustrate that the recombination events mediated by Xer1 recombinase are enhanced by sequences flanking the conserved 21-bp RS at the 5' end.

Recombination events between RS derived from different *vpma* genes. While the 21-bp RS sequence is completely conserved in the 5' UTR of all six *vpma* genes, the 5' flanking regions do not show significant homology within different *vpma* genes (10). Our previous results focused on recombination events between two RS that originated from the 5' UTR of the same gene, namely *vpmaY*. However, two *vpmaY* RS do not reflect the native alignment of (*vpma*) RS in the *M. agalactiae vpma* locus, where Xer1-mediated recombination occurs within the RS of two different *vpma* genes rather than that of the same *vpma* gene. VpmaY was shown to be expressed in *M. agalactiae* clone 55-5, a clonal variant of the type strain PG2 (10, 11), where the *vpmaY* gene is located downstream of the unique *vpma* promoter and therefore constitutes the expressed *vpma* gene. Our previous results indicated that a region of 36 bp flanking the 21-bp conserved sequence at the 5' end are required for the detection of Xer1-mediated inversion products via restriction analysis method after 20 h of *xer1* induction (Fig. 4B, lane 8). This 36-bp region is specific to the 5' UTR of the expressed *vpmaY* gene, and has no sequence homology with the 5' UTR of all other unexpressed *vpma* genes. As in pBADXerIR200Y both 200-bp RS originated from the 5' UTR of the expressed *vpmaY* gene, this configuration does not correspond with the actual recombination scenario that might be operating in the *vpma* locus of *M. agalactiae*. Inversions within the *vpma* locus occur between two inverted repeated RS of different *vpma* genes sharing the 21-bp region but have different nucleotides flanking this region at the 5' end. In order to verify if the two RS originating from different *vpma* genes also show similar results in the restriction analysis method, plasmid constructs carrying RS from

different *vpma* genes (Fig. 5) were constructed for further recombination experiments: in the first construct, namely pBADXerIR200Y/184U, one of the two *vpma*Y 200-bp RS within pBADXerIR200Y was replaced by a 184-bp *vpmaU* sequence (RS184U, Fig. 5A). As expected, inversions were detected after restriction analysis and gel electrophoresis of samples removed after 20 h of induction (Fig. 5B, lane 4). Similar inversion products were observed with construct pBADXerIR200Y/56X5' (Fig. 5B, lane 5) carrying a 200-bp *vpmaY* RS and a 56-bp RS of the *vpmaX* gene which covers the conserved 21-bp region and 35 bp flanking it at the 5' end (RS56X5', Fig. 5A). So far, all these inversions were observed in constructs where at least one of the RS was the 200-bp *vpmaY* RS. To verify if such inversions can be detected by restriction analysis even for those plasmids where both 200-bp *vpmaY*-RS are replaced by shorter versions, we constructed pBADXerIR53U5'/57Y5'. Here, a 57-bp *vpmaY*-RS, where the 21-bp region is extended by 36 bp at the 5'end (RS57Y5', Fig. 5A), and a 53-bp *vpmaU*-RS containing the 21 bp-region and 32 bp flanking it at the 5' end (RS53U5', Fig. 5A), were introduced alongside the *xer1* gene. When subjected to induction and restriction analysis, an inversion band of 4.6 kb was visible (Fig. 5B, lane 6). Such inversions between *vpmaU*-RS / *vpmaY*-RS and *vpmaX*-RS / *vpmaY*-RS not only reflect the recombination events actually operational in the *vpma* locus of the native *M. agalactiae* system, but also confirm our earlier results showing that the 5' flanking region of the 21-bp RS is indeed responsible for enhanced accumulation of recombination products.

Xer1 can mediate excisions between *vpma* RS in *M. agalactiae*. Having demonstrated excisions in *E. coli* by cloning two 200-bp *vpmaY* RS as direct repeats in plasmid pBADXerDR200Y (Fig. 3), the next step was to analyze if such excisions can occur in *M. agalactiae.* Since *vpma* genes are present in both orientations in the native *vpma* gene locus of *M. agalactiae* (4, 10), excisions between the direct repeated *vpma* RS are theoretically possible via the chromosomally encoded Xer1 recombinase. For analysing such excision events in the native *M. agalactiae* system, we developed a *lacZ*-based reporter system which was used to demonstrate Xer1-mediated excisions in *M. agalactiae* based on blue/white selection, as well by PCR analysis. The *lacZ* gene with its native Shine-Dalgarno sequence from *E. coli* was introduced into the left IS element of transposon Tn*4001*mod in pISM2062 (22) resulting in

recombinant plasmid pIL. Transformation of pIL into the wild-type *M. agalactiae* strain PG2, as well as into the *xer1*-disrupted PLMY (4) resulted in blue transformant colonies on SP4 agar plates supplemented with X-Gal (Fig. 6C). Interestingly, *lacZ* expression did not require the addition of a native promoter element upstream of the *lacZ* sequence indicating that transcription was driven by a transposon-based promoter. After this successful demonstration of *lacZ* as a reporter gene in *M. agalactiae*, plasmid pILDR (Fig. 6A) was constructed in which the promoterless *lacZ* gene was flanked by a *vpma*U-derived RS184U (Fig. 5A) at the 5' end and a *vpma*Y-derived RS200Y (Fig. 5A) at the 3' end, with both RS elements aligned as direct repeats. Transformation of pILDR into wild type PG2 strain indeed resulted in white colonies (Fig. 6C), as the *lacZ* gene was excised from the pILDR due to an excision event between the direct repeated *vpmaY*- and *vpmaU*-RS mediated by the chromosomal Xer1 recombinase (Fig. 6B). Transpositional integration of pILDR was confirmed in these white clones by Southern hybridization (data not shown) using a Tn-specific probe as described earlier (5). In contrast, pILDR transformants in the *xer1*-disrupted mutant strain PLMY still exhibited a faint blue-colony phenotype (Fig. 6C), as they lacked Xer1 recombinase to accomplish a similar *lacZ* excision (Fig. 6B). However, compared to the pIL transformants of PG2 and PLMY, these colonies showed reduced intensity of blue color (Fig. 6C). This might be due to the introduction of an extra 184-bp *vpmaU*-RS sequence upstream of the promoterless *lacZ* gene which leads to lower levels of *lacZ* transcription due to increased distance from the transposon promoter. These Xer1-mediated excisions were further confirmed by PCR analysis using primer 184Ubfw which anneals to the 184-bp *vpmaU*-RS, and primer ISR-f that anneals to a region upstream to the left IS element of Tn*4001*mod (Fig. 6B). Since, both primers anneal outside the region of recombination, the amplified product would verify the absence or presence of a *lacZ* gene excision event. As expected, PCR reactions using genomic DNA of PLMY transformed with pILDR resulted in amplification of just a single fragment of 3.5 kb (Fig. 6D, lane 3) corresponding to the unexcised *lacZ* sequence flanked by the two RS, indicating that no excision has occurred in this *xer*1-disruptant strain. On the contrary, a similar PCR reaction performed with genomic DNA obtained from pILDR PG2 transformants showed an additional band of 245-bp (Fig. 6D, lane 4) corresponding to the shortened sequence created after Xer1-mediated recombination of the *vpma*U- and *vpma*Y-RS that leads to the deletion of the

interjacent *lacZ* gene (Fig. 6B). Furthermore, sequencing of the 245-bp product displayed an expected hybrid site (RSUY), comprising the 5' region of the *vpma*U-RS, the 21-bp consensus sequence common for both RS, and the 3' region of the *vpma*Y RS. These results clearly demonstrated that Xer1-mediated excisions between direct repeated *vpma*-RS are feasible in *M. agalactiae*. The above data not only provide the first experimental proof of the *lacZ* reporter system being functional in *M. agalactiae*, but also confirm our earlier postulates regarding the Xer1 recombination system based on the results of the recombination experiments done in *E. coli*.

DISCUSSION

The results demonstrate that a consensus sequence (TTGATATTTATTAATAGATTT) of 21 bp present in the 5' UTR of all *vpma* genes is sufficient for Xer1-mediated inversion and that recombinational strand exchange occurs within this region. In site-specific recombination systems, core-RS constitute DNA sequences where recombinase binding and strand exchange take place. Typically, these core-RS regions exhibit dyad symmetry as two perfect or imperfect inverted repeats (11-13 bp) which act as recombinase binding elements surrounding a 6-8 bp overlap sequence within which strand cutting and exchange occurs (7, 12, 24). Alignment of the 21-bp RS of *M. agalactiae* with its reverse and complemented sequence identified an 8-bp palindromic central sequence (TATTAATA) but did not reveal any other significant dyad symmetry (Fig. 7). However, if the first 3 bases of the 21-bp site are excluded, similar alignment reveals an impressive level of dyad symmetry flanking the central 8-bp palindrome (Fig. 7). Generally, overlap sequences are non-palindromic and for the simplest site-specific recombination systems of the λ-integrase family, exemplified by the Cre recombinase of bacteriophage P1 and the Flp recombinase of *Saccharomyces cerevisiae*, this asymmetry is sufficient to provide polarity to the site (7). It has been well demonstrated that conversion of an asymmetric overlap region to a symmetric one leads to recombination events that have no polarity. A pair of such symmetric sites when placed on a DNA molecule, led to both excisions and inversions independent of their orientation as direct or inverted repeats (17, 29).

RS sequences involved in site-specific recombination systems of mycoplasmas show different levels of dyad symmetry. In *M. pulmonis,* the site-specific HvsR recombinase catalyzes inversions at two

distinct loci causing variation in the production of restriction-modification enzymes (*hsd* locus), as well as phase variation of surface lipoproteins (*vsa* locus). Comparison of the RS of these two different loci revealed no significant sequence similarity, where the dyad symmetry was very weak for *vsa* RS compared to the *hsd* RS (31). Recent studies in *M. penetrans* have demonstrated that site-specific recombination mediates *mpl* promoter inversions causing phase variation of individual *mpl* lipoprotein genes. When compared with RS of other site-specific recombinases, these inversions occur within a considerably short 12-bp inverted repeated sequence flanking the promoter region. Also, comparison of the inverted repeated sequence of individual *mpl* promoters revealed a consensus sequence of TAAYNNNDATTA, where nucleotides TAA and ATTA at both ends of the inverted repeated sequence resemble a dyad motif, whereas the central region differs from each other and might be responsible for preventing inappropriate recombination between different *mpl* promoters or for differences in the inversion frequencies of individual promoters (18). In general, it appears that the RS sequences of site-specific recombination systems of mycoplasmas do not exhibit comparable dyad symmetry as shown for other well-characterized site-specific recombination systems, and so it is difficult to predict the point of strand exchange and recombinase binding. Since several RS have been characterized in mycoplasmas, identification of binding sites for recombinase enzymes and detection of the crossover region involved during strand exchange may be instrumental for further understanding of such recombination reactions.

In vivo inversion experiments in *E. coli* using *vpma*-RS from the same, as well as from different *vpma* genes revealed that Xer1 can act in *trans*, and that additional nucleotides flanking the conserved 21-bp region at the 5' end lead to enhanced accumulation of recombination products. Indeed, many site-specific recombination systems require additional elements beyond the core-RS which typically include extra binding sites for the recombinase or accessory proteins required for efficient recombination (24). However, comparison of the 5' flanking nucleotides of the *vpma*-RS does not show any significant sequence identity among different *vpma* RS, except a box of 3 nucleotides (ATA) located 13-15 bases upstream of the 21-bp region. Comparing individual 5' flanking regions with each other shows sequence identity levels of up to 82.1% (including the 21-bp RS common for all *vpma* genes). Future studies may concentrate on the evaluation of inversion frequencies between the RS of

different *vpma* genes to assess if homology in this region is required to enhance the rate of recombination.

The newly developed excision assay based on the *lacZ* reporter gene successfully demonstrated excision events between two direct repeated *vpma*-RS in the *M. agalactiae* type strain PG2. The same orientation of *vpma* genes in the *vpma* locus (10) leads to direct repeated orientation of the RS, and any excision events between them would result in deletion of genetic material, including not only the *vpma* genes but also the unique *vpma* promoter, which is the key element for expression of these variable surface proteins. This might be critical for the survival of the pathogen in the host and resultant clones with shorter excised versions of the *vpma* locus might be selected against due to reduced fitness potential. Based on their result that some PG2 clones yield a very low proportion of smaller *vpma* fragments in addition to a high proportion of expected full-length amplicons during PCR amplification of the whole *vpma* locus, Glew et al (2002) have previously suggested that such excisions occur at a low frequency (10). Whether additional regulatory factors, as found in other bacterial site-specific recombination systems (2, 6, 16, 24, 30), control and favour *vpma* inversions above their excisions needs to be elucidated. Considering the minimal genome of *M. agalactiae*, it is possible that such stochastic excisions in a proportion of population could be the cost that the pathogen pays to maintain this antigenic variation machinery for overall population advantage.

ACKNOWLEDGEMENTS

This work was supported by grant P18668-B05 (to WJ and RR) of the Austrian Science Fund (FWF). The authors thank Martina Zimmermann for her excellent technical assistance and Dr. Michael Szostak (IBMH), Dr. Angela Witte (Department of Microbiology, Immunobiology and Genetics, University of Vienna), and Dr. Christine Citti (INRA-ENVT, Toulouse, France) for helpful discussions.

REFERENCES

1. **Barre, F. X., and D. J. Sherratt.** 2002. Xer site-specific recombination: promoting chromosome segregation, p. 149-161. *In* N. L. Craig, Craigie, R., Gellert, M., Lambowitz, A.M. (ed.), Mobile DNA II. ASM Press, Washington D.C.

2. **Bayliss, C.D.** 2009. Determinants of phase variation rate and the fitness implications of differing rates for bacterial pathogens and commensals. FEMS Microbiol. Rev. **33**: 504-520

3. **Bhugra, B., L. L. Voelker, N. Zou, H. Yu, and K. Dybvig.** 1995. Mechanism of antigenic variation in *Mycoplasma pulmonis*: interwoven, site-specific DNA inversions. Mol. Microbiol. **18:**703-714.

4. **Chopra-Dewasthaly, R., C. Citti, M. D. Glew, M. Zimmermann, R. Rosengarten, and W. Jechlinger.** 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. Mol. Microbiol. **67:**1196-1210.

5. **Chopra-Dewasthaly, R., M. Zimmermann, R. Rosengarten, and C. Citti.** 2005. First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn*4001*mod. Int. J. Med. Microbiol. **294:**447-453.

6. **Colloms, S. D., J. Bath, and D. J. Sherratt.** 1997. Topological selectivity in Xer site-specific recombination. Cell **88:**855-864.

7. **Duyne, G. D.** 2002. A structural view of tyrosine recombinase site-specific recombination, p. 93-113. *In* N. L. Craig (ed.), Mobile DNA II. ASM Press, Washington, D.C.

8. **Esposito, D., and J. J. Scocca.** 1997. The integrase family of tyrosine recombinases: evolution of a conserved active site domain. Nucleic Acids Res. **25:**3605-3614.

9. **Flitman-Tene, R., S. Mudahi-Orenstein, S. Levisohn, and D. Yogev.** 2003. Variable lipoprotein genes of *Mycoplasma agalactiae* are activated in vivo by promoter addition via site-specific DNA inversions. Infect. Immun. **71:**3821-3830.

10. **Glew, M. D., M. Marenda, R. Rosengarten, and C. Citti.** 2002. Surface diversity in *Mycoplasma agalactiae* is driven by site-specific DNA inversions within the *vpma* multigene locus. J. Bacteriol. **184:**5987-5998.

11. **Glew, M. D., L. Papazisi, F. Poumarat, D. Bergonier, R. Rosengarten, and C. Citti.** 2000. Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant variable surface proteins in *Mycoplasma agalactiae*. Infect. Immun. **68:**4539-4548.

12. **Grainge, I., and M. Jayaram.** 1999. The integrase family of recombinase: organization and function of the active site. Mol. Microbiol. **33:**449-456.

13. **Groth, A. C., and M. P. Calos.** 2004. Phage integrases: biology and applications. J. Mol. Biol. **335:**667-678.

14. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. **177:**4121-4130.

15. **Halbedel, S., and J. Stülke.** 2006. Probing in vivo promoter activities in *Mycoplasma pneumoniae*: a system for generation of single-copy reporter constructs. Appl. Environ. Microbiol. **72:**1696-1699.

16. **Hallet, B., and D. J. Sherratt.** 1997. Transposition and site-specific recombination: adapting DNA cut-and-paste mechanisms to a variety of genetic rearrangements. FEMS Microbiol. Rev. **21:**157-178.

17. **Hoess, R. H., A. Wierzbicki, and K. Abremski.** 1986. The role of the *loxP* spacer region in P1 site-specific recombination. Nucleic Acids Res. **14:**2287-2300.

18. **Horino, A., T. Kenri, Y. Sasaki, N. Okamura, and T. Sasaki.** 2009. Identification of a site-specific tyrosine recombinase that mediates promoter inversions of phase-variable *mpl* lipoprotein genes in *Mycoplasma penetrans*. Microbiology **155:**1241-1249.

19. **Janis, C., C. Lartigue, J. Frey, H. Wroblewski, F. Thiaucourt, A. Blanchard, and P. Sirand-Pugnet.** 2005. Versatile use of *oriC* plasmids for functional genomics of *Mycoplasma capricolum* subsp. *capricolum*. Appl. Environ. Microbiol. **71:**2888-2893.

20. **Jechlinger, W., J. Glocker, W. Haidinger, A. Matis, M. P. Szostak, and W. Lubitz.** 2005. Modulation of gene expression by promoter mutants of the lambdacI857/pRM/pR system. J. Biotechnol. **116:**11-20.

21. **Johnson, C. J.** 2002. Bacterial site-specific DNA-inversion systems, p. 230-271. *In* N. L. Craig, Craigie, R., Gellert, M., Lambowitz, A.M. (ed.), Mobile DNA II. ASM Press, Washington D.C.

22. **Knudtson, K. L., and F. C. Minion.** 1993. Construction of Tn*4001*lac derivatives to be used as promoter probe vectors in mycoplasmas. Gene **137:**217-222.

23. **Liu, L., K. Dybvig, V. S. Panangala, V. L. van Santen, and C. T. French.** 2000. GAA trinucleotide repeat region regulates M9/pMGA gene expression in *Mycoplasma gallisepticum*. Infect. Immun. **68:**871-876.

24. **Nash, H. A.** 1996. Site-specific recombination: integration, excision, resolution, and inversion of defined DNA segments, p. 2363-2376. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology. ASM Press, Washington, D.C.

25. **Razin, S., D. Yogev, and Y. Naot.** 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiol. Mol. Biol. Rev. **62:**1094-1156.

26. **Ron, Y., R. Flitman-Tene, K. Dybvig, and D. Yogev.** 2002. Identification and characterization of a site-specific tyrosine recombinase within the variable loci of *Mycoplasma bovis*, *Mycoplasma pulmonis* and *Mycoplasma agalactiae*. Gene **292:**205-11.

27. **Sadowski, P.** 1986. Site-specific recombinases: changing partners and doing the twist. J. Bacteriol. **165:**341-347.

28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

29. **Senecoff, J. F., and M. M. Cox.** 1986. Directionality in FLP protein-promoted site-specific recombination is mediated by DNA-DNA pairing. J. Biol. Chem. **261:**7380-7386.

30. **Sherratt, D. J., L. K. Arciszewska, G. Blakely, S. Colloms, K. Grant, N. Leslie, and R. McCulloch.** 1995. Site-specific recombination and circular chromosome segregation. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **347:**37-42.

31. **Sitaraman, R., A. M. Denison, and K. Dybvig.** 2002. A unique, bifunctional site-specific DNA recombinase from *Mycoplasma pulmonis*. Mol. Microbiol. **46:**1033-1040.

32. **Solsona, M., M. Lambert, and F. Poumarat.** 1996. Genomic, protein homogeneity and antigenic variability of *Mycoplasma agalactiae*. Vet. Microbiol. **50:**45-58.

33. **Tully, J. G.** 1995. Culture medium formulation for primary isolation and maintenance of mollicutes, p. 33-39. *In* S. Razin, Tully, J.G. (ed.), Molecular and Diagnostic Procedures in Mycoplasmology, vol. 1. Academic Press, San Diego.

FIGURE LEGENDS

FIG. 1. A three-primer PCR inversion assay for the detection of inversions within the two 21-bp RS conserved in the 5' UTR of all *vpma* genes. (A) Schematic representation of inversion of plasmid pBADXerRS21x2 carrying two inverted repeated 21-bp RS (RS21, indicated by bold black arrows) along with the *xer1* gene, shown in comparison with (B) plasmid pBADRS21x2, which carries the same RS but cannot undergo inversions as it lacks a *xer1* gene and thus acts as a negative control. Primers P1, P2 and P4 annealing to the *araC* sequence (regulatory gene of L-arabinose operon), the *bla* sequence (ampicillin resistance gene) and the region adjacent to the *ori* (pBR322 origin), respectively, are indicated by thin black arrows. (C) Agarose gel electrophoresis of PCR products at different stages of arabinose induction. Presence of a 1 kb P1-P2 amplicon corresponds to inversion events in *E.coli* between the two RS21 sequences only upon Xer1 induction at 6 and 20 h (lanes 7 and 8) for pBADXerRS21x2, and is absent for the uninduced overnight sample (lane 6). The latter, as well as both the induced (lanes 4 and 5) and uninduced (lane 3) samples of control plasmid pBADRS21x2, show only the 1.4 kb P1-P4 product amplified from the sequence of unrecombined plasmids. Lane 2: No DNA template control; Lanes 1 and 9: molecular size marker (1 kb ladder, Invitrogen).

FIG. 2. Restriction analysis of the inversion events occurring between two inverted repeated 200-bp RS in *E. coli*. (A) Schematic representation of inversion of plasmid pBADXerIR200Y carrying two inverted repeated 200-bp RS (RS200Y, indicated by bold black arrows) along with the *xer1* gene (white arrow). Induction of *xer1* results in inversion of the DNA fragment flanked by the two RS200Y resulting in plasmid pBADXerIR200Y IV where the *Hind*III and *Eco*RV sites are located close to each other. *ori*, pBR322 origin; *bla*, ampicillin resistance gene; *araC*, regulatory gene of L-arabinose

operon. (B) Agarose gel electrophoresis of *Hind*III- and *Eco*RV-digested recombination products obtained at different time points. Two fragments of 3.6 kb (OP1) and 1.9 kb (OP2) that correspond to the original unrecombined plasmid are present in all samples. An inversion fragment of 4.9 kb (IV) is visible after 2 h (lane 4), 4 h (lane 5) and 6 h (lane 6) of *xer1* induction, whereas it is absent in the uninduced cells grown overnight (lane 2) and in the sample taken at the start of induction (lane 3). Lanes 1 and 7: molecular size marker (1 kb ladder, Invitrogen). (C) Sequence of the RS200Y fragment obtained from the *vpmaY* gene of *M. agalactiae*. The bold letters represent the 21-bp conserved sequence found in the 5' UTR of all *vpma* genes.

FIG. 3. Xer1 mediates excisions between two 200-bp RS. (A) Schematic representation of excision of plasmid pBADXerDR200Y carrying two direct repeated 200-bp RS (RS200Y, indicated by bold black arrows) along with the *xer1* gene (white arrow). Induction of *xer1* results in excision of the DNA-fragment flanked by the two direct repeated RS200Y resulting in two recombination products: miniplasmid (mp) and minicircle (mc). *ori*, pBR322 origin; *bla*, ampicillin resistance gene; *araC*, regulatory gene of L-arabinose operon. (B) An inverted image of agarose gel electrophoresis of supercoiled (sc) recombination products. Supercoiled miniplasmid (sc mp) and supercoiled minicircle (sc mc) are visible 2 h (lane 4), 4 h (lane 5) and 6 h (lane 6) after *xer1* induction beside the band corresponding to the unrecombined original plasmid (sc op). Recombination products are not visible in the uninduced cells grown overnight (on, lane 2) and in the sample taken at the start of induction (0h, lane 3). Lanes 1 and 7: molecular size marker (1 kb ladder, Invitrogen). (C) An inverted image of agarose gel electrophoresis of *Hind*III- and *Xba*I-linearized (lin) recombination products confirms the sizes of the original unrecombined plasmid pBADXerDR200Y (X/H op, 5.5 kb) and the minicircle and miniplasmid (lin mc, 2.3 kb and lin mp, 3.2 kb) excised out of it upon *xer1* induction. Lanes 1 and 7: molecular size marker (1 kb ladder, Invitrogen).

FIG. 4. Sequences flanking the 21-bp RS at the 5' end enhance the amount of Xer1 inversion products. One of the two 200-bp *vpmaY* RS in pBADXerIR200Y was replaced by shorter versions of different lengths and flanking regions, namely RS21Y (in pBADXerIR21Y, the white box represents the 21-bp

conserved 5' UTR), RS31Y3' (in pBADXerIR31Y3'), RS56Y3' (in pBADXerIR56Y3'), RS111Y (in pBADXerIR111Y), and RS57Y5' (in pBADXerIR57Y5') (A) to determine the minimal RS which gives detectable amounts of inversion products (IV) during restriction analysis and agarose gel electrophoresis (B). Samples were digested with *Hind*III and *Eco*RV after 20 h of *xer1* induction. Plasmid pBADXerIR200Y showed a 4.9 kb inversion band between 200Y/200Y RS after induction (lane 3) and served as a positive control, whereas its uninduced overnight sample was negative for an inversion band (lane 2) and only showed bands that correspond to the unrecombined parent plasmid (OP1 and OP2). Similarly, no inversion was detectable between 200Y/21Y RS (lane 4), 200Y/31Y3' RS (lane 5) and 200Y/56Y3' RS (lane 6), whereas appearance of 4.8 kb (lane 7) and 4.75 kb (lane 8) bands indicate inversion events between 200Y/111Y RS and 200Y/57Y5', respectively. Lanes 1 and 9: molecular size marker (1 kb ladder, Invitrogen).

FIG. 5. Inversion events between different *vpma* RS. Plasmids carrying RS sequences derived from two different *vpma* genes were constructed to reflect the native recombination events operative in *M. agalactiae*, and transformed into *E. coli* to check for inversion events. (A) Schematic representation of the RS elements used in the different plasmid constructs: RS200Y and RS57Y5' (from *vpmaY*), RS184U and RS53U5' (from *vpmaU*) and RS56X5' (from *vpmaX*); white box depicts the 21-bp conserved region (B) Agarose gel electrophoresis of *Hind*III and *Eco*RV digests of different samples removed after 20 h of *xer1* induction. The inversion bands (IV) and the bands corresponding to the original unrecombined plasmid (OP1 and OP2) are indicated on the left margin. Plasmid pBADXerIR200Y showed a 4.9 kb inversion band between 200Y/200Y RS after induction (lane 3) and served as a positive control, whereas its uninduced overnight sample was negative for an inversion band (lane 2) and only showed bands that correspond to the unrecombined parent plasmid (OP1 and OP2). Inversion bands of 4.9 kb (lane 4), 4.75 kb (lane 5) and 4.6 kb (lane 6) were respectively observed for the newly constructed pBADXerIR200Y/184U, pBADXerIR200Y/56X5' and pBADXerIR53U5'/57Y5' plasmids where the two RS originated from different *vpma* genes as indicated. Lanes 1 and 7: molecular size marker (1 kb ladder, Invitrogen).

FIG. 6. Demonstration of Xer1-mediated excisions in *M. agalactiae.* (A) Illustration of plasmid pILDR used for studying Xer1-mediated excisions in *M. agalactiae*: the *lacZ* gene is flanked by a 184-bp *vpma*U RS and a 200-bp *vpma*Y RS (RS184U and RS200Y, respectively, black arrowheads) aligned as direct repeats within the left Insertion Sequence of transposon Tn*4001*mod (IS256L). *ori*, pBR322 origin; *bla*, ampicillin resistance gene; *gm*, gentamicin resistance gene. (B) Schematic representation of the genomic integration of pILDR in *M. agalactiae* type strain PG2 (PG2 pILDR) and the *xer1*-disruptant PLMY (PLMY pILDR): Xer1 recombinase (white ellipse) mediates excision between RS184U and RS200Y resulting in deletion of the interjacent *lacZ* sequence in PG2, but not in PLMY. Primers 184Ubfw and ISR-f (thin black arrows) used for detecting excisions via PCR and their corresponding amplicons (thin black lines) are indicated for both PG2 and PLMY. (C) Colony phenotype of *lacZ* transformants: parent plasmid pIL, which carries the *lacZ* gene without direct repeated RS (not shown), when transformed into PG2 (PG2 pIL) and PLMY (PLMY pIL) resulted in intense blue colonies on SP4 agar plates containing X-Gal. Introduction of plasmid pILDR in PG2 (PG2 pILDR) displayed white colonies, indicating that the *lacZ* gene was lost by site-specific excision between the direct repeated RS mediated by the *M. agalactiae*-encoded Xer1 recombinase (see Fig. 6B). In contrast, excision is absent in the *xer1*-mutant PLMY indicated by blue colony formation (PLMY pILDR). (D) Agarose gel electrophoresis of PCR reactions verifying excision events in *M. agalactiae*: Primers 184Ubfw and ISR-f (indicated in Fig. 6B), both annealing outside the region of recombination, were used to detect excision of the *lacZ* gene. Genomic DNA of PLMY pILDR (lane 3) enables amplification of only a 3.5 kb fragment, corresponding to the *lacZ* sequence, whereas PCR reactions using genomic DNA of PG2 pILDR transformants (lane 4) also displayed a 245-bp excision fragment corresponding to the hybrid RSUY created after excison between RS184U and RS200Y, in addition to the 3.5 kb *lacZ* fragment. Lane 2: No DNA template control. Lanes 1 and 5: molecular size marker (1 kb ladder, Invitrogen).

FIG. 7. Dyad symmetry in the 21-bp sequence conserved in the 5' UTR of *vpma* genes. (A) Nucleotide sequence of the conserved 21-bp region, and (B) the aligned sequence of 21-bp and 18-bp regions (where the first three nucleotides of 21 bp are excluded) with the respective reverse complemented

sequence. The central palindromic sequence is in bold letters and regions with dyad symmetry are indicated by asterisks.

TABLE 1. Oligonucleotides

Oligonucleotide name	Sequence (5' to 3') [a]
RSmcsX	AATTAATCTAGAGCAATTAGTAGAAGATTGTAGCG
RSmcsH	AATGTAAAGCTTGGCCATTGAAGCAACTGATCCAAG
Rsd1cla	AATTAAATCGATGCAATTAGTAGAAGATTGTAGCG
Rsd1nsi	AATGTAATGCATGGCCATTGAAGCAACTGATCCAAG
Rsd2cla	AATGTAATCGATGCCCATTGAAGCAACTGATCCAAG
Rsd2nsi	AATTAAATGCATGCAATTAGTAGAAGATTGTAGCG
RecATGET28	AACATTCCATGGTAGAAACCTTTATCAC
XerrvSma	GCTCGACCCGGGCTACTTACTATTAAGC
P1	CAGACAATTGACGGCTTGACG
P2	TTCTCTTACTGTCATGCCATCCG
P4	TGGACTCTTGTTCCAAACTTG
Ol1new2sen	CTAGATTGATATTTATTAATAGATTTA
Ol1new2asen	AGCTTAAATCTATTAATAAATATCAAT
Oligo#2sense	CTAGTTGATATTTATTAATAGATTTATAAAGCATTA
Oligo#2asense	AACTATAAATAATTATCTAAATATTTCGTAATTCGA
Oligo#3sense	CTAGTTGATATTTATTAATAGATTTATAAAGCATTTTT AAGGCTATTTTATAGCCTTTAAA
Oligo#3asense	AACTATAAATAATTATCTSAATATTTCGTAAAAATTCC GATAAAATATCGGAAATTTTCGA
pBADoligo#4fw	GATCAATCTAGAGCTATTAATTTAGCACTTAATAC
pBADoligo#4rv	AATGTCAAGCTTTCATAAATTTATCCTTTC
Ol9sen	CTAGAGCTATTAATTTAGCACTTAATACATCATATAATAAATTGATATT ATTAATAGATTTA
Ol9asen	AGCTTAAATCTATTAATAAATATCAATTTATTATATGATGTATTAAGTG CTAAATTAATAGCT
PrimerRSU	AATTGCTCTAGAAAGTTTAATCAAAACTTAACG
OligoRSXsen	CTAGATTTAGTAAGCAGTCGCTAAGCATATACTTGTACTTTTGATATTT ATTAATAGATTTA

OligoRSXasen	AGCTTAAATCTATTAATAAATATCAAAAGTACAAGTATATGCTTAGCG ACTGCTTACTAAAT
OligoRSUsen	CTAGAGATGTTTTTATATTCTCATAAACTCACTGTTTTTGATATTTATTA ATAGATTTA
OligoRSUasen	AGCTTAAATCTATTAATAAATATCAAAAACAGTGAGTTTATGAGAATA TAAAAACATCT
RS21NsiI	CGCCAAATCTATTAATAAATATCAAATGCA
RS21NarI	TTTGATATTTATTAATAGATTTGG
LacfwBN	GCACTAGGATCCCCATGGAGGAAACAGCTATGACCATGATTA
LacrvSB	ATCACGCCCGGGAGATCTTTATTATTTTTGACACCAGACCAACTG
184Ubfw	TCATGAGGATCCAAGTTTAATCAAAACTTAAC
184Unrv	ATACAACCATGGGGCCATTGAAGCAACTGATC
200YBfw	TACTAGAGATCTGGCCATTGAAGCAACTG
200YBrv	AAGCTTAGATCTGCAATTAGTAGAAGATTG
ISR-f	TATAACGCGTGATAAAGTCCGTATAATTGTG

[a] Restriction sites (underlined) were introduced into primers

FIG. 1

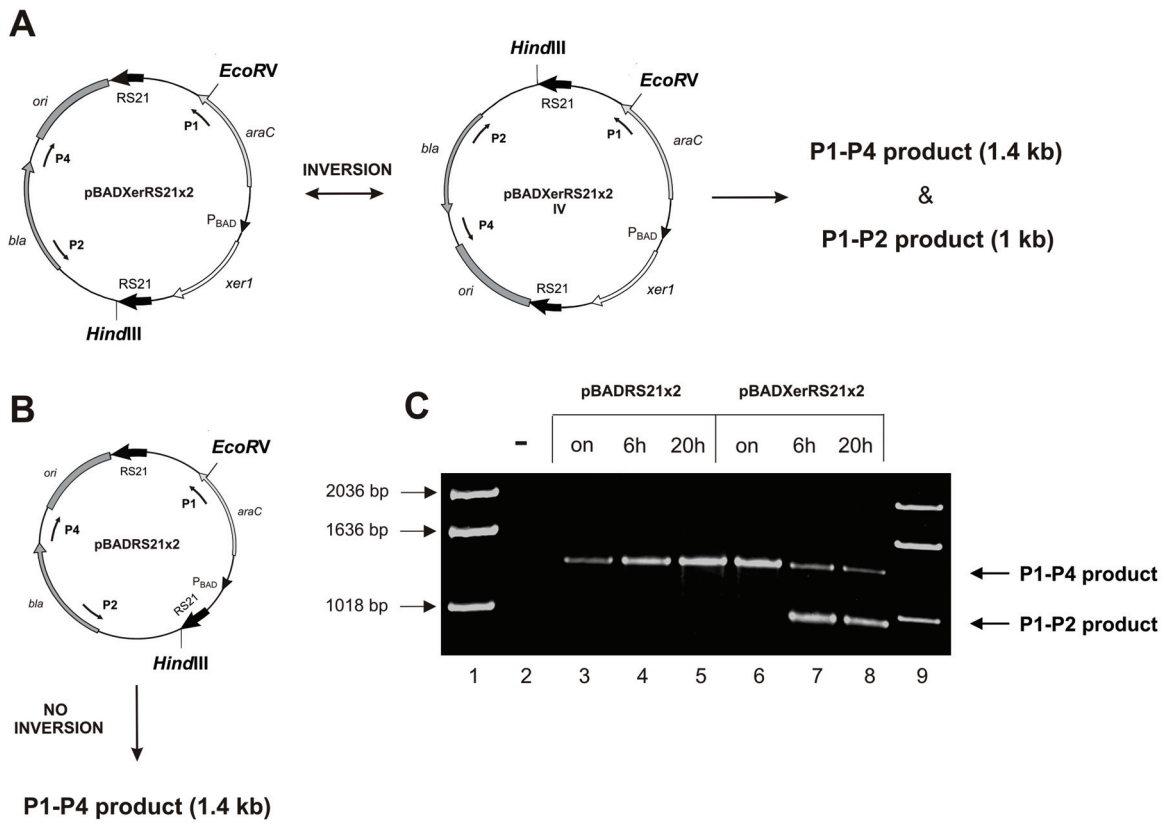
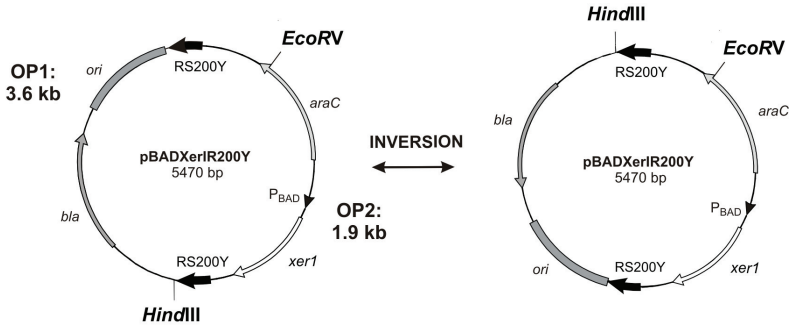
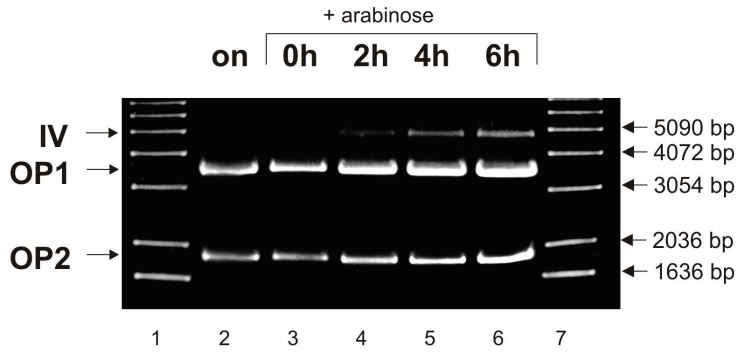

A
EcoRV
RS21
ori
P1
araC
P4
pBADXerRS21x2
P BAD
bla
P2
RS21
xer1
HindIII
INVERSION
HindIII
EcoRV
RS21
bla
P2
P1
araC
pBADXerRS21x2
IV
P4
P BAD
ori
RS21
xer1
P1-P4 product (1.4 kb)
&
P1-P2 product (1 kb)
B
EcoRV
ori
RS21
P1
araC
P4
pBADRS21x2
P BAD
bla
P2
RS21
HindIII
NO
INVERSION
P1-P4 product (1.4 kb)
C
pBADRS21x2
pBADXerRS21x2
-
on
6h
20h
on
6h
20h
2036 bp
1636 bp
1018 bp
P1-P4 product
P1-P2 product
1
2
3
4
5
6
7
8
9

FIG. 2

A



B



C

```
RS200Y GCAATTAGTAGAAGATTGTAGCGTTTTAATTAAATGGCTTAATTTGCTATTAATTTAGCACTTAATACAT 70
RS200Y CATATAATAAATTGATATTTATTAATAGATTTATAAAGCATTTTTAAGGCTATTTTATAGCCTTTAAAGA 140
RS200Y AAGGATAAATTTATGAAAAAATCAAAGTTTTTATTACTTGGATCAGTTGCTTCAATGGCC           200
        SD    (Start Codon)
```

FIG. 3

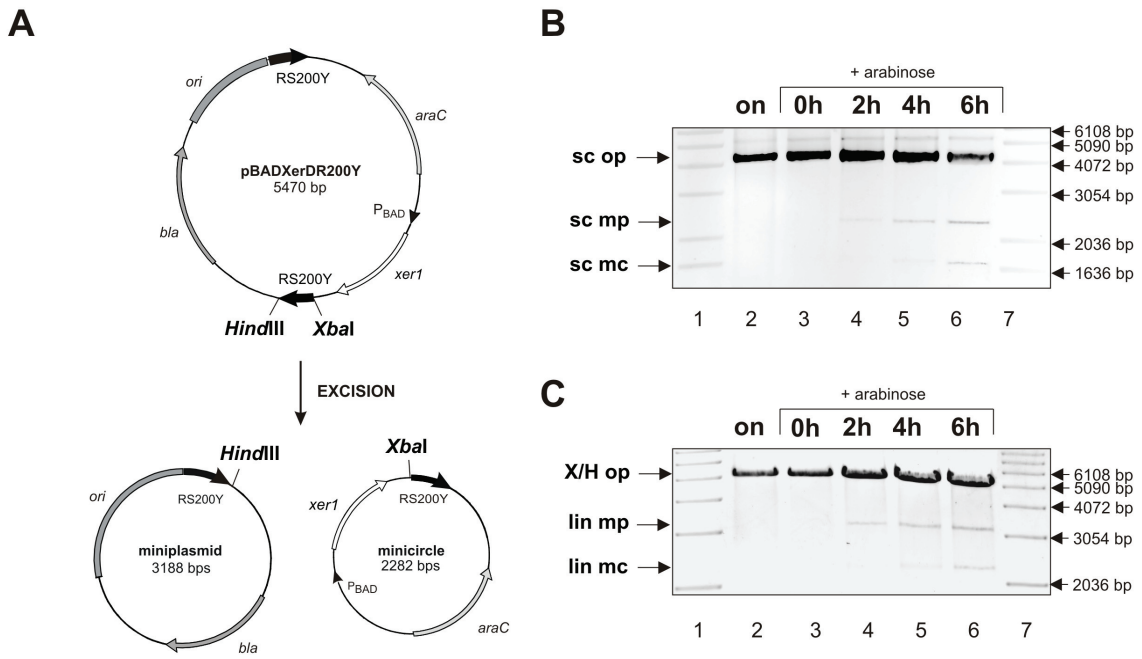

A
ori
RS200Y
araC
pBADXerDR200Y
5470 bp
PBAD
bla
RS200Y
xer1
HindIII
XbaI
EXCISION
HindIII
XbaI
ori
RS200Y
miniplasmid
3188 bps
bla
xer1
RS200Y
minicircle
2282 bps
PBAD
araC
B
+ arabinose
on
0h
2h
4h
6h
sc op
sc mp
sc mc
6108 bp
5090 bp
4072 bp
3054 bp
2036 bp
1636 bp
1 2 3 4 5 6 7
C
+ arabinose
on
0h
2h
4h
6h
X/H op
lin mp
lin mc
6108 bp
5090 bp
4072 bp
3054 bp
2036 bp
1 2 3 4 5 6 7

FIG. 4

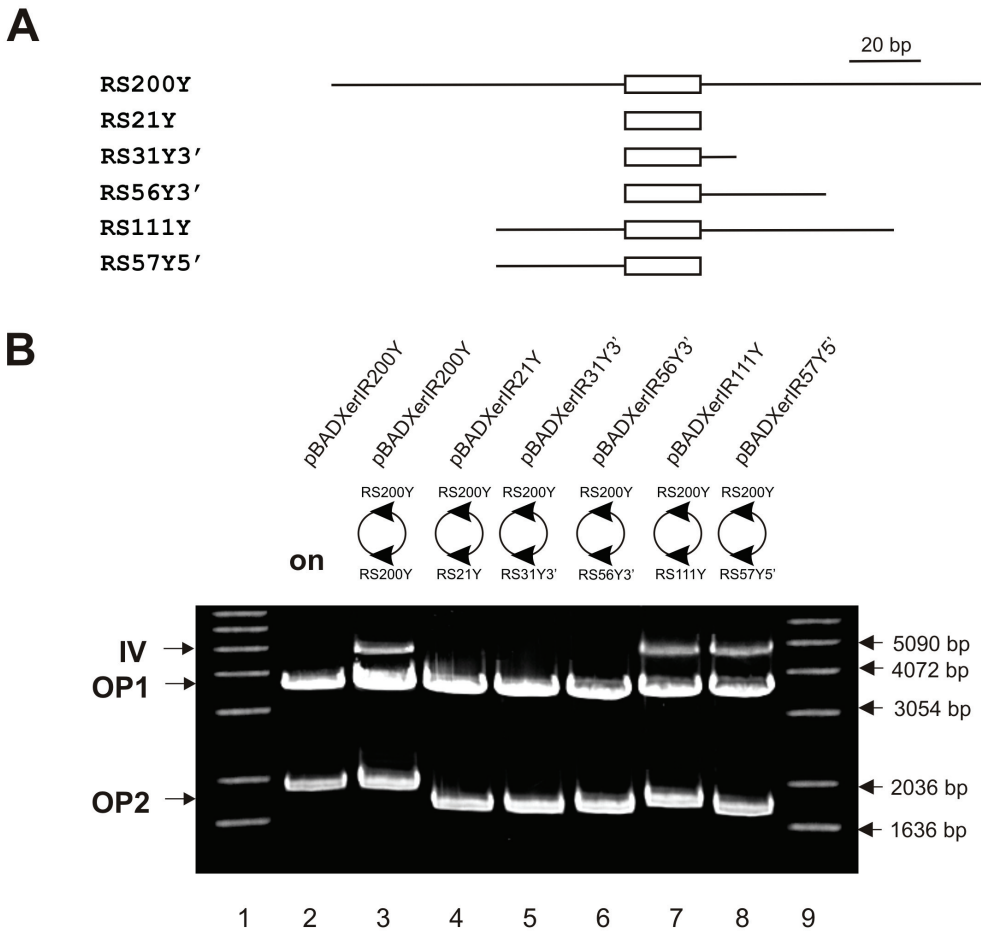

A
20 bp
RS200Y
RS21Y
RS31Y3′
RS56Y3′
RS111Y
RS57Y5′
B
pBADXerIR200Y
pBADXerIR200Y
pBADXerIR21Y
pBADXerIR31Y3'
pBADXerIR56Y3'
pBADXerIR111Y
pBADXerIR57Y5'
on
RS200Y
RS200Y
RS200Y
RS200Y
RS200Y
RS200Y
RS200Y
RS21Y
RS31Y3'
RS56Y3'
RS111Y
RS57Y5'
IV
OP1
OP2
5090 bp
4072 bp
3054 bp
2036 bp
1636 bp
1 2 3 4 5 6 7 8 9

FIG. 5

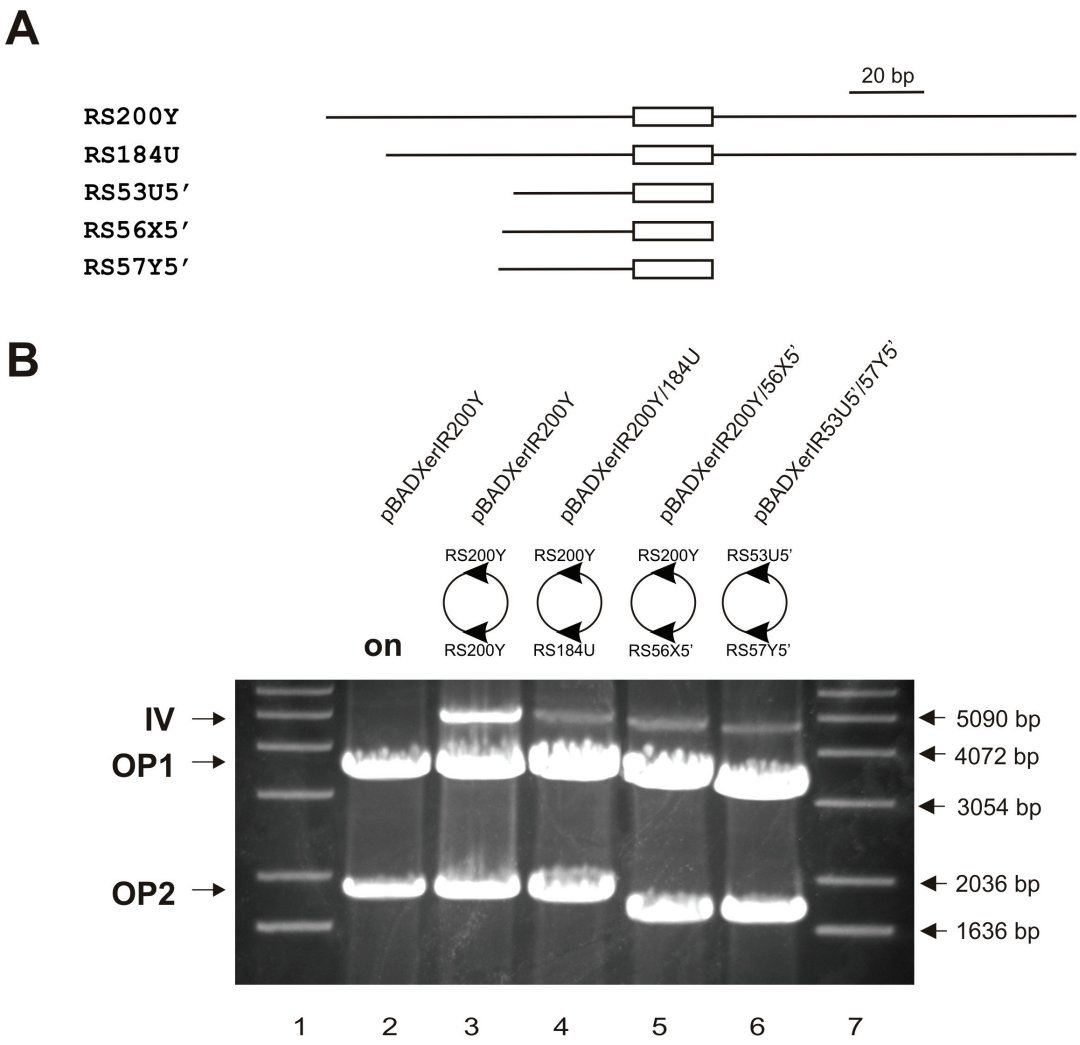

A
20 bp
RS200Y
RS184U
RS53U5′
RS56X5′
RS57Y5′
B
pBADXerIR200Y
pBADXerIR200Y
pBADXerIR200Y/184U
pBADXerIR200Y/56X5′
pBADXerIR53U5′/57Y5′
on
RS200Y
RS200Y
RS200Y
RS53U5′
RS200Y
RS184U
RS56X5′
RS57Y5′
IV
OP1
OP2
5090 bp
4072 bp
3054 bp
2036 bp
1636 bp
1
2
3
4
5
6
7

FIG. 6

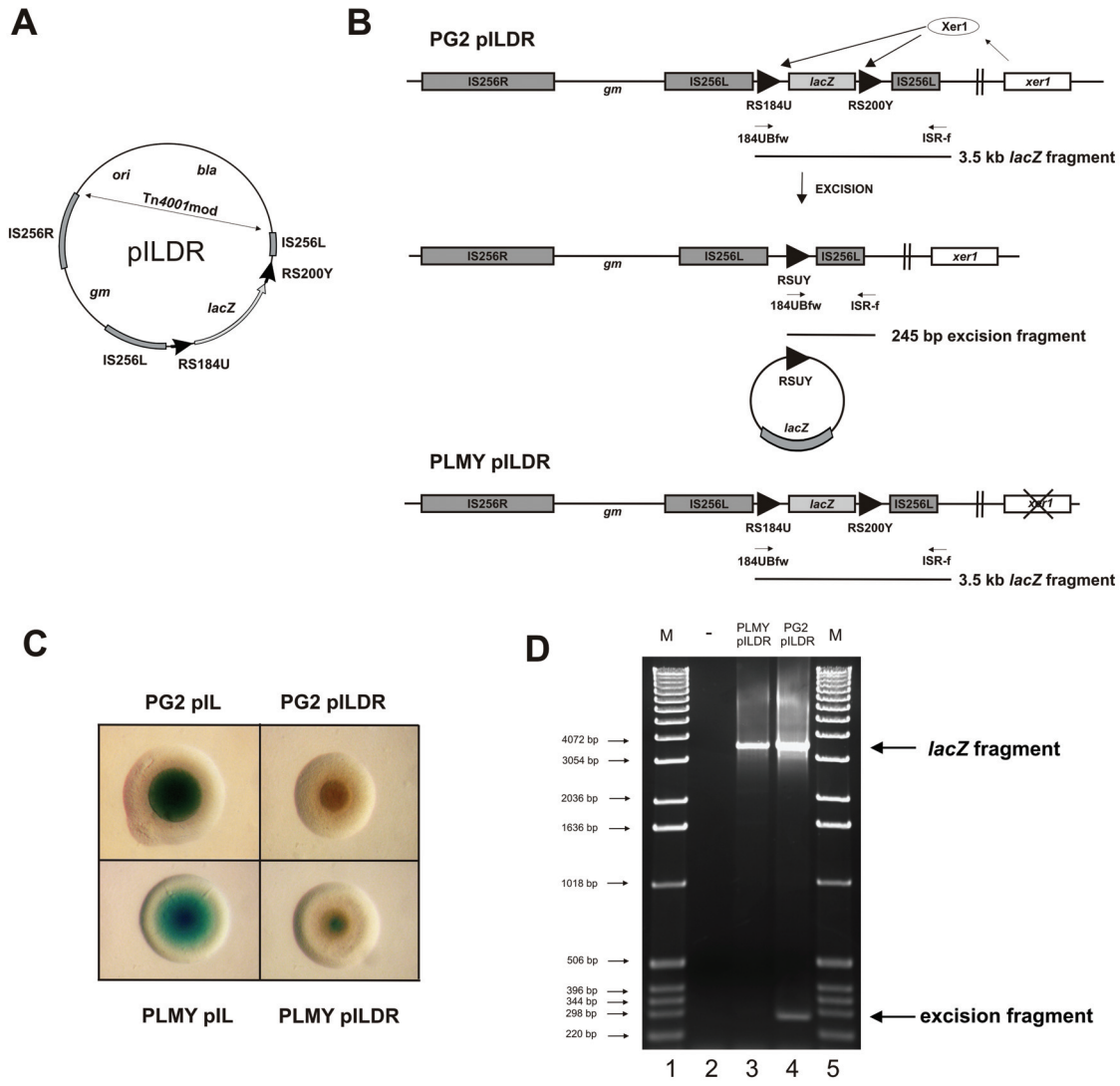
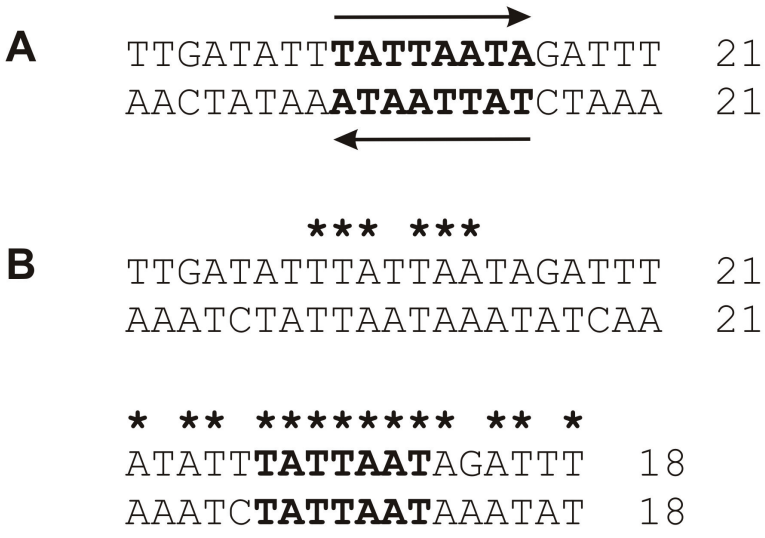

A
ori
bla
Tn4001mod
IS256R
pILDR
IS256L
RS200Y
gm
lacZ
IS256L
RS184U
B
PG2 pILDR
Xer1
IS256R
gm
IS256L
lacZ
IS256L
xer1
RS184U
RS200Y
184UBfw
ISR-f
3.5 kb lacZ fragment
EXCISION
IS256R
gm
IS256L
IS256L
xer1
RSUY
184UBfw
ISR-f
245 bp excision fragment
RSUY
lacZ
PLMY pILDR
IS256R
gm
IS256L
lacZ
IS256L
xer1
RS184U
RS200Y
184UBfw
ISR-f
3.5 kb lacZ fragment
C
PG2 pIL
PG2 pILDR
PLMY pIL
PLMY pILDR
D
M
-
PLMY pILDR
PG2 pILDR
M
4072 bp
3054 bp
2036 bp
1636 bp
1018 bp
506 bp
396 bp
344 bp
298 bp
220 bp
lacZ fragment
excision fragment
1
2
3
4
5

FIG. 7


A
TTGATATTTATTAATAGATTT 21
AACTATAAATAATTATCTAAA 21
*** ***
B
TTGATATTTATTAATAGATTT 21
AAATCTATTAATAAATATCAA 21
* ** ******** ** *
ATATTTATTAATAGATTT 18
AAATCTATTAATAAATAT 18

5.2 Collection of Data: Xer1-independent Mechanisms of Vpma Phase Variation are Triggered by the Presence of Vpma-specific Antibodies

INTRODUCTION

Mycoplasma agalactiae, the main etiologic agent of the syndrome Contagious Agalactia in sheep and goats, exhibits high-frequency antigenic variation of its major immunodominant surface lipoproteins, the so-called Vpmas (variable proteins of *M. agalactiae*) (Flitman-Tene et al., 2003; Glew et al., 2000; 2002). Six *vpma* genes, encoded by the pathogenicity island-like *vpma* multigene locus, were demonstrated to undergo phase variation by specific DNA inversions mediated by a site-specifc recombinase (Xer1) of the λ-integrase family encoded adjacent to the *vpma* genes (Flitman-Tene et al., 2003; Glew et al., 2000; 2002). Functional knock-out of the *xer1* gene in the *M. agalactiae* type strain PG2 has clearly demonstrated that Xer1 recombinase is essential for site-specific recombinations within the *vpma* locus (Chopra-Dewasthaly et al., 2008). In the *xer1*-disrupted Vpma phase-locked mutants (PLMs) further *vpma* switching by site-specific DNA inversions was abolished and cells were locked in an expression phase where only one *vpma* gene is steadily expressed (Chopra-Dewasthaly et al., 2008).

The aim of the present study was to evaluate if Xer1-independent mechanism may provide an additional level of Vpma antigenic diversity in the *M. agalactiae* type strain PG2 to survive in the animal host in absence of Xer1-mediated *vpma* inversions. Previously, our group had performed an experimental infection of sheep using a mixture of two PLMs, namely PLMU and PLMY (manuscript in preparation). Screening of the Vpma expression patterns of two clones isolated from the animals revealed that PLMs, which were stable for several generations in vitro had undergone Vpma switching. Genomic characterization of the *vpma* gene profiles of both clones demonstrated that DNA rearrangements other than Xer1-mediated site-specific inversions occurred in the *vpma* locus, including the generation of hybrid *vpma* genes, as well as *vpma* gene duplication and deletion, which lead to altered Vpma phenotypes. Recent studies on the *M. agalactiae* field strain 5632 have also identified some *vpma* genes displaying a mosaic structure, which are most likely results of similar recombination events (Nouvel et al., 2009).

For a limited number of *Mycoplasma* species, in vitro assays have been used to know if specific antibodies directed towards predominantly expressed surface antigens cause variability of the same as a result of immune pressure. In the bovine pathogen *M. bovis* it was demonstrated that monoclonal antibodies directed towards the major variable surface antigens (Vsps), as well as serum antibodies from immunized or experimentally infected calves can repress expression of the target proteins or induce switching to antigenically different proteins (Le Grand et al., 1996). In *M. hyorhinis* immune pressure assays were useful to evaluate the role of Vlp surface proteins in determining susceptibility to growth inihibitory effects of host antibodies (Citti et al., 1997). Similarly, cultivation of *M. hominis* in the presence of monoclonal antibodies directed against the 135-kDa surface exposed antigen Lmp1 led to the selection of escape mutants that were shown to contain deletions of repetitive domains within the corresponding *lmp1* gene. However, these mutations were reported to be associated with spontaneous agglutination events, indicating that repetitive domains of Lmp1 may be important for repulsion of cells (Jensen et al., 1995).

In order to know if Vpma-specific antibodies are the factors that trigger Vpma switching in *M. agalactiae*, and especially to test the hypothesis that Xer1-independent Vpma phenotypic changes were induced and selected by the immune defences of the host, we designed an immune pressure assay to reconstruct the results obtained from the animal experiments. Briefly, two PG2 derived strains, the clonal variant 55-5 predominantly expressing VpmaY (Glew et al., 2000; 2002) and the VpmaY expressing *xer1*-deficient mutant PLMY, which is unable to undergo further Vpma switching (Chopra-Dewasthaly et al., 2008), were cultivated in the presence of a complement inactivated monospecific rabbit polyclonal antibody (pAb) recognizing the VpmaY protein (Chopra-Dewasthaly et al., 2008). In the presence of the α-Y pAb immune pressure, both strains were able to switch Vpma phenotypes although to very different extents and using different molecular mechanisms. As expected, high-frequency Vpma switching in the wild-type strain 55-5 is governed by Xer1-mediated site-specific inversions, whereas strain PLMY tries to overcome this immune pressure by a low frequency Xer1-independent mechanism resulting in the generation of novel hybrid *vpma* gene variants. Similar mechanism and *vpma* hybrids were also demonstrated for the *xer1*-disrupted clones isolated from animals after an experimental infection with PLMs (manuscript in preparation). Together, these results

clearly demonstrate that Xer1-independent *vpma* recombinations occur both in vitro, as well as in vivo in the *M. agalactiae* type strain PG2 and mediate Vpma surface variation under selection pressure of the host-induced immune response.

MATERIALS AND METHODS

Bacterial strains and culture conditions. In this study the *M. agalactiae* type strain PG2 (Solsona et al., 1996), the PG2 derived clonal variant 55-5 (Glew et al., 2000) and the *xer1*-deficient mutants PLMY and PLMU (Chopra-Dewasthaly et al., 2008) were used. PG2 and 55-5 were propagated at 37°C in modified SP4 broth and agar plates (Chopra-Dewasthaly et al., 2005). For cultivation of PLMs, SP4 was supplemented with tetracycline at a concentration of 2 μg ml^{-1}.

Immune pressure assay. Exponential phase cultures of 55-5 and PLMY (approximately 10^9 CFU/ml) were ten-fold serially diluted to achieve cultures with approximately 10^6 CFU/ml. To 180 μl of the diluted culture 20 μl of heat-inactivated (56°C for 30 min) α-Y pAb (Chopra-Dewasthaly et al., 2008) was added (10% [vol/vol] concentration in standard medium). Control samples contained 20 μl PBS instead of the pAb. Cultures were incubated for 10 days at 37°C. To monitor growth, aliquots of cultures were plated at different time points on SP4 agar plates. CFU values were estimated by counting colonies under the stereomicroscope (Nikon). Mean CFU/ml values were calculated from three independent experiments.

Colony immunoblot and Western blot analysis. Screening of the Vpma expression patterns was carried out by colony immunoblot analysis essentially as described elsewhere (Chopra-Dewasthaly et al., 2008). Briefly, nitrocellulose membranes were placed on the freshly grown colonies on agar plates for 5 min, detached and washed two times in TBS buffer (10 mM Tris, 154 mM NaCl, pH 7.4) before an overnight incubation at 4°C in pAb specific for the VpmaY protein (Chopra-Dewasthaly et al., 2008). After three washing steps in TBS buffer containing 0.05% Tween 20 (Roth) for 10 min each, membranes were incubated for 2 h at room temperature in 1:2000 diluted swine anti-rabbit IgG conjugated to horseradish peroxidase (DakoCytomation, Denmark). After three washing steps in TBS

buffer, blots were developed in 4-chloro-1-naphthol (Bio-Rad) and hydrogen peroxide. The reaction was stopped after 20 min by washing membranes in water.

Western blotting was accomplished by standard sodium dodecylsulphate-polyacrylamide gel electrophoresis using whole cell extracts of *M. agalactiae* cultures as described previously (Chopra-Dewasthaly et al., 2008). Cell pellets were resuspended in PBS and boiled at 95°C for 5 min under reducing conditions. Proteins were separated in 10% polyacrylamide gels containing 3% (w/v) urea. Proteins were electrophoretically transferred to nitrocellulose membranes (Roth) using blotting buffer (48 mM Tris, 39 mM Glycine, 0.037% SDS and 20% Methanol) and immunostained by the same protocol as described for colony immunoblots.

PCR reactions, DNA manipulation and Southern hybridization. Digoxigenin (DIG)-labeling (Roche) of *vpma*-specific gene probes by PCR was carried out according to the manufacturer's recommendations using PG2 genomic DNA as template and a set of following primer pairs (oligonucleotides are summarized in Table 1) for individual *vpma*-specific gene probes: U2F and Urev1 (*vpmaU*), C1F and C1R (*vpmaV*), WDIGfw and WDIGrv (*vpmaW*), X1F and X1R (*vpmaX*), Y3F and Y3R (*vpmaY*), Z1F and Z2R (*vpmaZ*). PCR cycling conditions were as follows: 1 cycle of initial denaturation for 3 min at 94°C, 30 cycles of 95°C for 1 min, 57°C (*vpmaU*, *vpmaV*, *vpmaX*, *vpmaY*, *vpmaZ*) or 65°C (*vpmaW*) for 1 min, followed by 30s (*vpmaU*, *vpmaV* , *vpmaX*, *vpmaY*, *vpmaZ*) or 1 min (*vpmaW*) at 72°C and a final extension step for 5 min at 72°C.

Mycoplasma genomic DNA was isolated by the method described previously (Chopra-Dewasthaly et al., 2005). For *vpma*-specific Southern blots genomic DNA was cut with appropriate restriction endonucleases: *Hind*III for hybridization with *vpmaW*-specific probe, *Hind*III and *Xba*I for *vpmaU*-, *vpmaV*-, *vpmaX*- and *vpmaZ*-specific probes and *Pst*I for *vpmaY*-specific probe. Digested DNA was subjected to agarose gel electrophoresis and DNA fragments were transferred to nylon membranes (Roth) using a standard protocol (Sambrook et al., 1989). Hybridization with DIG-labeled probes and washing under stringent conditions followed by non-radioactive detection was carried out according to the manufacturer's recommendations (Roche). Southern blot analysis confirming *xer1*-disruption was carried out as described previously (Chopra-Dewasthaly et al., 2008).

Cloning and sequencing of *vpma* loci. In order to define the *vpma* configuration of the escape variants PLM18, PLM16 and X-EV, *Cla*I digested genomic DNA was self ligated and transformed into *E. coli* DH10B as described (Chopra-Dewasthaly et al., 2008). Tetracycline and ampicillin-resistant transformants were isolated and subjected to restriction and PCR analysis. Plasmid preparations of recombinant plasmids were used for DNA sequencing. Sequencing was carried out by LGC Genomics (Germany). Primers used for sequencing are summarized in Table 1.

RESULTS AND DISCUSSION

PLMs switch over to new Vpma phenotypes in the native host via Xer1-independent mechanisms.

Generation of *xer1*-deficient PLMs has greatly facilitated the possibilty not only to study the role of individual Vpma proteins but also of alternative Vpma switching mechanisms which might act in the absence of the Xer1 recombinase (Chopra-Dewasthaly et al., 2008). To address this issue, previously an equimolar mixture of two PLMs (PLMU and PLMY) was used in an experimental infection of sheep. Initial *vpma* gene configuration of both PLMY and PLMU is demonstrated in Fig. 1A whereby the single *vpma* promoter lies upstream of *vpmaY* and *vpmaU* genes thus leading to the stable 'locked' expression of VpmaY and VpmaU, respectively (Chopra-Dewasthaly et al., 2008). However, colony immunoblot analysis of some animal reisolates demonstrated Vpma phenotypes other than VpmaY and VpmaU. Two such clones, namely PLM16 and PLM18, when analysed via Western and colony immunoblotting clearly revealed that they had undergone Vpma switching to antigenically different Vpma proteins even in absence of the Xer1 protein. PLM16 shows reactivity exclusively with the α-W pAb, whereas PLM18 reacts only with the α-X pAb and both are negative for the expression of VpmaY and VpmaU, the original Vpma phenotype of PLMY and PLMU from which they originated (data not shown). In both clones, the absence of a functional *xer1* gene was verified by Southern blot analysis (Fig. 7C, lanes 4 and 5) (Chopra-Dewasthaly et al., 2008). To further understand the molecular basis of Vpma switching in these *xer1*-disrupted clones, the *vpma* gene configuration of both clones was examined (Fig. 1 and Fig. 2).

PLM18 has undergone intergenic *vpma* recombination to generate hybrid *vpma* genes and altered Vpma phenotypes. Southern blot analysis using individual *vpma*-specific gene probes (Fig. 7B, lanes 4), as well as sequencing of the PLM18 *vpma* locus demonstrated that its *vpma* configuration is similar to that of PLMY with the exception that two hybrid genes, *vpmaYX'* and *vpmaXY'* (Fig. 1B), are present instead of *vpmaX* and *vpmaY* of the type strain PG2. Located downstream to the single identified promoter, *vpmaXY*' constitutes the expressed gene in PLM18 (Fig. 1B). Comparison of the coding sequence of the hybrid *vpmaXY*' gene (Fig. 1C) with the original *vpma* sequences of the PG2 clonal variant 55-5 and PLMY indicates that an intergenic recombination event has occurred at a 38-bp sequence (Fig. 1C, bold letters) that is common to both *vpmaX* and *vpmaY* gene sequences. In the hybrid gene *vpmaXY*', the sequence upstream of this 38-bp homologous region was found to be 100% identical with the *vpmaY* gene, whereas the downstream region showed complete identity with the sequence of *vpmaX* (Fig. 1C). Recombination at this 38-bp homologous sequence not only resulted in the generation of two hybrid genes, but also led to inversion of the promoter that resulted in the alteration of the observed Vpma phenotype depending on the specific Vpma antibody epitopes present in the downstream region (Fig. 1B). Since various conserved regions are repeated within different *vpma* genes, it would be not unlikely that generation of hybrid genes by homologous recombination represents a common mechanism for immune evasion. This view is supported by the fact that similar recombination events were also demonstrated in the *vsp* gene locus of the bovine pathogen *M. bovis*, the closest phylogenetic relative of *M. agalactiae*. Genetic analysis of clonal isolates derived from the *M. bovis* type strain PG45 revealed that intergenic recombination between two closely related members of the *vsp* gene family, *vspA* and *vspO*, led to the formation of the hybrid gene *vspC* (Lysnyansky et al., 2001).

PLM16 has undergone complex *vpma* rearrangements leading to duplication and deletion of specific *vpma* genes. In contrast to PLM18, which contains hybrid *vpma* genes generated by intergenic recombination within homologous *vpma* regions, examination of the PLM16 *vpma* locus revealed a much more complex recombination scenario. Phenotypic expression of VpmaW in this clone correlates well with the sequencing analysis that reveals the presence of *vpmaW* gene located

downstream of the promoter (Fig. 2). Sequencing data also revealed the duplication of two *vpma* genes, namely *vpmaX* and *vpmaW,* as also supported by Southern blot analysis whereby two bands are observed with both *vpmaW* and *vpmaX* probes when the genomic DNA is digested with restriction enzymes that cut outside these genes, respectively (Fig. 7B, lane 5, *vpmaW* and *vpmaX* probes). Additionally, the sequence of PLM16 *vpma* locus showed that three genes, namely *vpmaY*, *vpmaU* and *vpmaZ,* are completely absent (Fig. 2). In accordance, no hybridization signal was observed during Southern blot analysis with probes specific for *vpmaU*, *vpmaY* and *vpmaZ* genes (Fig. 7B, lane 5, *vpmaU, vpmaY* and *vpmaZ* probes) clearly verifying the absence of these three genes. However, it is difficult to reconstruct the hierarchy of rearrangements that occurred in PLM16 that led to the final conformation seen in Fig. 2. The *vpma* configuration present in this clone is predicted to be a result of recombination events that occurred in PLMY. Although speculative, duplication of *vpmaX* and *vpmaW* could have placed the *vpmaW* gene downstream of the promoter, whose position is unaltered compared to PLMY, and simultaneously deleted the genomic fragment carrying *vpmaU*, *vpmaY* and *vpmaZ* (Fig. 2).

In both the animal reisolates, significant *vpma* reshuffling has occurred under immune pressure in the native host, which clearly demonstrates the requirement of antigenic variation systems as a key feature for suvival during natural *M. agalactiae* infection. Despite Vpma phase variation by site-specific recombination, homologous recombination between conserved *vpma* sequences may alternatively represent an important immune evasion mechansim.

In vitro immune pressure assay demonstrates bactericidal and growth inhibitory effect of Vpma-specific pAbs on *M. agalactiae*. To evaluate the role of Vpma-specific antibody response as a factor that triggers Vpma surface variation, as well as to investigate possible growth inhibitory effects of anti-Vpma pAbs, an in vitro immune pressure assay was established, whereby PG2 clonal variant 55-5 (Glew et al., 2002) and *xer1*-disrupted mutant PLMY (Chopra-Dewasthaly et al., 2008), showing predominant and exclusive expression of VpmaY, respectively, were grown in presence of complement-inactivated VpmaY-specific α-Y pAb. The obvious difference between 55-5 and PLMY is that 55-5 is capable of switching to alternative Vpma phenotypes due to Xer1-mediated site-specific

vpma inversions (Glew et al., 2002; Chopra-Dewasthaly et al., 2008), whereas the *xer1*-deficient strain PLMY is blocked in the *vpmaY* expression phase and is unable to express any other Vpma protein (Chopra-Dewasthaly et al., 2008). Both strains were cultivated at 37°C in the presence of α-Y pAb over a period of 10 days. Cultures incubated in the presence of PBS served as negative controls. To monitor growth, aliquots of cultures were plated at the time of antibody addition and additionally after 1, 2, 4, 7, 8 and 10 days of growth. CFU values were estimated by counting colonies under a stereomicroscope. Representative growth of 55-5 and PLMY in the presence and absence of the pAb is shown in Fig. 3.

A significant decline in the PLMY population was observed after one day incubation in medium containing α-Y pAb, as concluded from the CFU values which dropped below the starting inoculum size of 10^6 cfu/ml (Fig. 3A). After this initial decline, PLMY recovers and shows some growth although this rate of growth is significantly lower than the control PLMY cultures grown in absence of α-Y pAb. This indicates that initial decline in CFU is a result of a strong immune pressure eliminating the majority of cells expressing VpmaY and eventual selection and growth of a small subpopulation of escape variants. We predicted that these cells are likely to stop VpmaY expression and then become capable of stable but reduced growth. In contrast, a slight initial delay in growth was observed for the wild-type strain 55-5 in α-Y pAb, which was then fully overcome at the end of two days (Fig. 3B). Since 55-5 is capable of Xer1-mediated Vpma phase variation, this initial delay may very well correlate with a time frame required for switching off the expression of the *vpmaY* gene and to switch on to new alternative Vpma phenotypes. These growth irregularities observed for both strains in the presence of VpmaY-specific antibodies suggest that Vpma phenotypic switching mechanisms serve significant roles in evading the host immune response during natural or experimental *M. agalactiae* infections.

Clonal variant 55-5 escapes the growth inhibitory effect of α-Y pAb by switching to alternative non-VpmaY phenotypes via high-frequency Xer1-mediated *vpma* recombinations. To determine, whether the α-Y pAb could trigger Vpma switching in the wild-type strain 55-5, Vpma expression patterns of samples removed at the initial stage of immune pressure (2 days of incubation in α-Y pAb)

were analyzed by colony immunobloting. As shown in Fig. 4, growth of 55-5 in the presence of α-Y pAb induces switching from the predominant VpmaY phenotype to the expression of other five alternative Vpma proteins as indicated by the appearance of sectored colonies with α-Y pAb during colony immunostaining and the simultaneous appearance of sectored and positive colonies with other five Vpma-specific pAbs (Fig. 4). In contrast, control samples of 55-5 grown in PBS instead of α-Y pAb display a positive phenotype when immunostained with α-Y pAb and predominantly negative phenotypes with the remaining five pAb (Fig. 4). This clearly shows that the generation of Vpma antigenic variants in 55-5 is induced by α-Y pAb and that it is only because of this high-frequency Vpma switching to alternative non-VpmaY phenotypes that the clone 55-5 completely escapes the growth damaging effects of α-Y pAb as seen in Fig. 3. This could very well reflect the immune evasion capablity of wild-type *M. agalactiae* in the native immunocompetent host.

Huge growth deficits due to α-Y pAb are partially overcome by PLMY by repressing the expression of VpmaY. Examination of PLMY growth rates revealed a significant reduction of the PLMY bacterial population in the presence of the α-Y pAb (Fig. 3A). Colony immunoblots of samples removed at 2 days of growth in α-Y pAb show that the majority of PLMY colonies now display a VpmaY negative phenotype indicating repression of the previous sole expression of VpmaY (Fig. 5A). In previous studies, analysis of the genomic organization of the *vpma* locus of PLMY revealed that *vpmaY* gene is the expressed gene located downstream of the unique promoter followed by the *vpmaZ* gene in the same transcriptional orientation (Chopra-Dewasthaly et al., 2008). As both genes are separated by a weak Rho-independent terminator structure, a weak read-through transcription of VpmaZ was detected by Western blot analysis (Chopra-Dewasthaly et al., 2008). However, in this study we used a different VpmaZ-specific α-Z pAb that revealed positive immunostaining of PLMY even during colony immunoblotting (data not shown) probably due its higher sensitivity compared to the α-Z pAb used in the previous study. Interestingly, although in presence of α-Y pAb PLMY switches off VpmaY expression, yet it still showed positive immunostaining with α-Z pAb (data not shown). This suggested that inhibition of VpmaY expression might be a result of a mutational event within *vpmaY* gene causing premature termination of VpmaY translation without affecting the read-

through transcription of *vpmaZ*. And indeed, sequencing of such selected VpmaY negative clones revealed intragenic deletion within the *vpmaY* gene (Fig. 5B) corresponding to the loss of α-Y pAb recognition epitope. Originally, in PG2 and PLMY, the *vpmaY* gene consists of two large repeats of 558 bp, out of which the second repeat (RY2', Fig. 5B) is prematurely terminated due to the insertion of a stop codon (Glew et al, 2002; Chopra-Dewasthaly et al, 2008). However, in the sequenced VpmaY negative clones of PLMY, the 558 bp full length repeat (RY1, Fig. 5B) is found to be missing and the *vpmaY* gene constitutes only a single prematurely terminated version of the repeat, namely RY2'. This leads to the expression of a truncated VpmaY protein that lacks the epitope recognized by the α-Y pAb (Fig. 5B), which was originally encoded on the full length 558 bp repeat in PLMY. This very well correlates with the VpmaY negative phenotype observed during colony immunoblotting using α-Y pAb and clearly shows that intragenic *vpmaY* deletions constitute the molecular mechanism that led to VpmaY negative phenotype in PLMY when grown under α-Y pAb immune pressure. In contrast, PLMY samples incubated without the α-Y pAb show expression of both proteins, VpmaY (Fig. 5A) and VpmaZ and do not show any intragenic deletions in *vpmaY* sequence (data not shown). Furthermore, the α-Y pAb-induced VpmaY negative phenotype of PLMY clones was tested to be stable even after 20 passages in liquid media without any further selection in α-Y pAb (data not shown).

PLMY can generate low frequency 'escape' variants in α-Y pAb. In order to assess if incubation in α-Y pAb can also trigger PLMY to express alternate Vpma phenotypes like 55-5, cultures exposed to the α-Y pAb selection pressure for 2 days were screened via colony immunoblotting to detect possible 'escape' variants. For this, colony immunostaining was carried out with antibodies specifically recognizing VpmaU, VpmaV, VpmaW and VpmaX using agar plates seeded with a large number of colonies. Screening with α-Z pAb was omitted because of the likely read-through expression of VpmaZ in PLMY (Chopra-Dewasthaly et al., 2008). Indeed, a single fully stained colony designated X-EV (VpmaX expressing Escape Variant) could be identified on blots immunostained with VpmaX-specific α-X pAb (Fig. 5C). Southern blot analysis verified disruption of the *xer1* gene in the X-EV indicating that the Vpma-switchover has occurred in the absence of Xer1-mediated recombination

(Fig. 7C, lane 3). Colony blots of negative control cultures incubated with PBS instead of the α-Y pAb, did not show any such 'switchovers', neither with α-X pAb nor with any of the other three tested Vpma antisera (α-V pAb, α-W pAb and α-U pAb) (data not shown). To obtain a pure culture of the X-EV, three successive rounds of filter cloning were carried out. Verification of the VpmaX phenotype was then accomplished by colony immunoblotting and Western blotting using all six α-Vpma pAbs, which clearly demonstrated that the X-EV shows reactivity exclusively with the α-X pAb, but not with any other α-Vpma pAb (Fig. 5D). The above data clearly reveal that *M. agalactiae* is capable of generating Vpma phase variants via alternative Xer1-independent mechanisms, especially under the selection pressure of specific Vpma antibodies. Although the in vitro rate of generation of such Vpma variants is significantly low especially when compared to the Xer1-mediated Vpma phase variation of 55-5, yet it is possible that such 'escape' variants are generated at a higher frequency in the native immunocompetent host.

Molecular characterization of the in vitro α-Y pAb induced X-EV escape variant of PLMY reveals a strong correlation with 'escape' variants generated in vivo during experimental PLM sheep infections. To further understand the molecular basis of the Xer1-independent escape mechanism which resulted in the in vitro alteration of the Vpma phenotype, the gene configuration of the *vpma* locus in the X-EV was examined. DNA sequencing revealed the presence of two hybrid *vpma* genes, designated as *vpmaYX* and *vpmaXY* (Fig. 6A), replacing the orginial *vpmaX* and *vpmaY* genes. The *vpmaYX* gene constitutes the expressed gene downstream of the *vpma* promoter, whereas the adjacent encoded *vpmaXY* is transcriptionally silent (Fig. 6A). Both hybrid genes share a common region of 42 bp, which is also present in the wild-type *vpmaX* and *vpmaY* genes, but the flanking regions are identical either with the *vpmaX* or *vpmaY* gene. Alignment of the expressed *vpmaYX* gene with *vpmaY* and *vpmaX* is shown in Fig. 6B. The sequence upstream of the common 42-bp region is identical with the sequence of the *vpmaY* gene, whereas the downstream adjacent region shows 100% identity with the *vpmaX* sequence (Fig. 6B). However, compared to the initial *vpma* locus of PLMY, the promoter orientation has also changed (Fig. 6A). Intergenic recombination could be further shown by Southern blot analysis. During intergenic recombination, a *Hind*III site present in the 5' region of

the original *vpmaY* gene has switched to the 5' region of the hybrid *vpmaYX* gene. *Hind*III and *Xba*I digestion of genomic DNA of the X-EV followed by hybridization with a *vpmaX*-specific probe detects a 0.5 kb *Hind*III fragment, which is not observed in PLMY, clearly verifying that intergenic recombination has occurred between *vpmaX* and *vpmaY* genes (Fig. 7B). This mechanism is similar to that described in escape variant PLM18, which was isolated from the animals, with the exception, that another conserved region common for both genes, *vpmaY* and *vpmaX*, was involved in recombination. Since similar recombination events also occurred in the animals, we propose that Vpma antibody response is likely to play a major role during immune evasion in the native host. The generation of hybrid genes by intergenic recombination between homologous *vpma* regions represents an alternative immune evasion mechanism that is predicted to act at frequencies much lower than Xer1-mediated recombination, which was demonstrated to occur at frequencies of 10^{-2}-10^{-3} per cell per generation (Chopra-Dewasthaly et al., 2008).

REFERENCES

Chopra-Dewasthaly, R., C. Citti, M. D. Glew, M. Zimmermann, R. Rosengarten, and W. Jechlinger. 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. Mol. Microbiol. **67**:1196-1210.

Chopra-Dewasthaly, R., M. Zimmermann, R. Rosengarten, and C. Citti. 2005. First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn*4001*mod. Int. J. Med. Microbiol. **294**:447-453.

Citti, C., M. F. Kim, and K. S. Wise. 1997. Elongated Versions of Vlp Surface Lipoproteins Protect *Mycoplasma hyorhinis* Escape Variants from Growth-Inhibiting Host Antibodies. Infect. Immun. **65**:1773-1785.

Flitman-Tene, R., S. Mudahi-Orenstein, S. Levisohn, and D. Yogev. 2003.Variable lipoprotein genes of *Mycoplasma agalactiae* are activated in vivo by promoter addition via site-specific DNA inversions. Infect. Immun. **71**:3821-3830.

Glew, M. D., L. Papazisi, F. Poumarat, D. Bergonier, R. Rosengarten, and C. Citti. 2000. Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant variable surface proteins in *Mycoplasma agalactiae*. Infect. Immun. **68**:4539-4548.

Glew, M. D., M. Marenda, R. Rosengarten, and C. Citti. 2002. Surface diversity in *Mycoplasma agalactiae* is driven by site-specific DNA inversions within the *vpma* multigene locus. J. Bacteriol. **184**:5987-5998.

Jensen, L. T., S. Ladefoged, S. Birkelund, and G. Christiansen. 1995. Selection of *Mycoplasma hominis* PG21 deletion mutants by cultivation in the presence of monoclonal antibody. Infect. Immun. **63**:3336–3347.

Le Grand, D., M. Solsona, R. Rosengarten, and F. Poumarat. 1996. Adaptive surface antigen variation in *Mycoplasma bovis* to the host immune response. FEMS Microbiol. Lett. **144**:267-275.

Lysnyansky, I., Ron, Y., Sachse, K., and Yogev, D. 2001. Intrachromosomal recombination within the *vsp* locus of *Mycoplasma bovis* generates a chimeric variable surface lipoprotein antigen. Infect. Immun. **69**: 3703-3712.

Nouvel, L. X., M. Marenda, P. Sirand-Pugnet, E. Sagne, M. Glew, S. Mangenot, V. Barbe, A. Barre, S. Claverol, and C. Citti. 2009. Occurrence, plasticity, and evolution of the *vpma* gene family, a genetic system devoted to high-frequency surface variation in *Mycoplasma agalactiae*. J. Bacteriol. **191**:4111-4121.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Solsona, M., M. Lambert, and F. Poumarat. 1996. Genomic, protein homogeneity and antigenic variability of *Mycoplasma agalactiae*. Vet. Microbiol. **50**:45-58.

FIGURE LEGENDS

FIG. 1. Generation of hybrid *vpma* genes as a result of immune evasion in the native host. (A) *vpma* configuration of PLMY and PLMU constituting the initial inoculum used for experimental infection of sheep: *vpma* genes are illustrated as large arrows in different colours, the unique promoter is shown as a bent arrow. PLMY shows exclusive expression of *vpmaY*, whereas PLMU exclusively expresses *vpmaU* found downstream of the unique promoter. (B) DNA sequence analysis of *vpma* configuration of PLM18 isolated from animals experimentally infected with an equimolar mixture of PLMY and PLMU: inversion of the promoter (crossed broken lines) and the generation of two hybrid *vpma* genes, *vpmaYX'* and *vpmaXY'* (indicated in blue/red colours), is illustrated. The *vpmaXY'* gene constitutes the expressed gene located downstream of the *vpma* promoter, whereas the *vpmaYX'* gene is transcriptionally silent. VpmaX phenotype of PLM18 can be explained by the fact that the coding sequence corresponding to the epitope recognized by α-X pAb is present at the 3' end of the expressed *vpmaXY'* gene (black bar). (C) Sequence alignment of the hybrid *vpmaXY'* gene of PLM18 with the sequence of the original *vpmaX* and *vpmaY* genes: a 38-bp region common to both *vpma* genes (bold letters) is identified in the hybrid *vpmaXY'* gene. The upstream region of the common 38-bp region in *vpmaXY'* shows complete sequence identity with the *vpmaY* gene, whereas the downstream region is identical with the *vpmaX* gene, clearly indicating that the homologous 38-bp region was involved in an intergenic recombination event between *vpmaX* and *vpmaY*, which led to promoter inversion and generation of hybrid *vpmaXY'* gene.

FIG. 2. DNA sequencing of the *vpma* locus of PLM16 reveals gene duplications and deletions. The *vpmaW* gene is found downstream of the promoter (bent arrow) and correlates with the VpmaW phenotype of PLM16. DNA sequence analysis revealed the duplication of *vpmaX* and *vpmaW*, whereas *vpmaY*, *vpmaU* and *vpmaZ* are deleted. The coding sequence corresponding to the epitope recognized by α-W pAb is shown as a black bar below the *vpmaW* gene. Hypothetical gene duplication and deletion events, that could have occurred in PLMY and led to the *vpma* configuration of PLM16 are illustrated.

FIG. 3. Growth of 55-5 and PLMY in the presence of VpmaY-specific α-Y pAb. Representative growth of the *xer1*-mutant PLMY (A) and the wild-type strain 55-5 (B) in SP4 medium supplemented with α-Y pAb (triangles) and PBS (squares) is shown

FIG. 4. High-frequency Vpma modulation in PG2 clonal variant 55-5 allows it to escape the damaging effects of VpmaY-specific α-Y pAb. Colony immunoblot analysis of PG2 clone 55-5 grown for 2 days in absence (panel PBS) and in presence (panel α-Y pAb) of VpmaY-specific polyclonal antiserum. In PBS, clone 55-5 continues to predominantly express VpmaY and shows hardly any positive immunostained colonies with the other five monospecific Vpma pAbs, namely α-U, α-V, α-W, α-X and α-Z, as designated below each column. In presence of α-Y pAb, 55-5 escapes its growth inhibitory effect (i) by repressing VpmaY expression (majority of colonies show negative or sectored colony immunoblot patterns with α-Y pAb) and (ii) by switching over to the other five Vpma phenotypes at a high frequency, as evident from the positive and sectored colony immunostaining patterns with α-U, α-V, α-W, α-X and α-Z pAbs.

FIG. 5. VpmaY-specific antibody represses VpmaY expression and triggers low-frequency generation of alternate Vpma variants in the *xer1*-deficient PLMY clone. (A) α-Y pAb colony immunostaining patterns of PLMY incubated in the presence of α-Y pAb in comparison with control samples grown in PBS. Colonies corresponding to samples grown in α-Y pAb show a predominant negative phenotype (pink colour after staining with Ponceau S) indicating that VpmaY expression was repressed in the presence of the antibody, whereas colonies of control samples display a VpmaY positive phenotype (purple colour with CN staining). (B) Schematic representation of VpmaY (expressed in PLMY control samples incubated in PBS) including the full-length repeat RY1 and the premature terminated repeat RY2' in comparison with the truncated version of VpmaY (expressed in the presence of α-Y pAb) where the RY1 repeat is missing; the leader sequence (L) of VpmaY is illustrated as a black box, the epitope recognized by α-Y pAb is shown as a black bar. (C) Detection of an 'escape' variant (X-EV) under the growth inhibitory effect of α-Y pAb as observed during α-X pAb colony immunostaining. X-EV demonstrates positive staining (indicated by an arrow) whereas most other

colonies are negative and are visible as pink colonies only after Ponceau S staining. (D) Colony immunoblotting and Western blot analysis of the filter-cloned X-EV 'escape' variant using six monospecific Vpma pAbs, namely α-U, α-V, α-W, α-X and α-Z, as designated for each panel. X-EV displays positive staining only with the α-X pAb, but not with any of the remaining five α-Vpma pAbs. Whole cell extracts of PG2 cultures, expressing all six Vpma proteins, were used as positive controls in Western blot analysis. Strain PLMY, exclusively expressing VpmaY, and strain PLMU, exclusively expressing VpmaU, served as respective negative controls.

FIG. 6. Genotypic analysis of the *vpma* locus of the α-Y pAb induced X-EV clone. (A) *vpma* gene configuration of the X-EV based on DNA sequencing is shown in comparison to that of PLMY constituting the initial *vpma* configuration before incubation in the α-Y pAb. *vpma* genes are illustrated as large arrows in different colours, the unique promoter is shown as a bent arrow. Incubation in α-Y pAb resulted in the generation of two hybrid *vpma* genes (*vpmaXY* and *vpmaYX*, indicated in blue/red colours) which are products of recombination within a 42-bp homologous region (vertical black lines) within the original *vpmaX* and *vpmaY* genes of PLMY. Recombination leads to inversion (crossed broken lines) of the promoter resulting in expression of the hybrid *vpmaYX* gene. The coding sequence of the epitope recognized by the α-VpmaX pAb (black bar) is located at the 3' end of the *vpmaYX* gene. (B) Sequence alignment of the original *vpmaX* and *vpmaY* genes with the hybrid *vpmaYX* gene of the X-EV. Bold letters represent the 42-bp sequence common for both genes, *vpmaX* and *vpmaY*, which is also present in *vpmaYX*. The adjacent 5' sequence shows sequence identity with *vpmaY*, whereas the downstream 3' region is identical with *vpmaX*, indicating that the hybrid gene has been generated by homologous recombination within this homologous region.

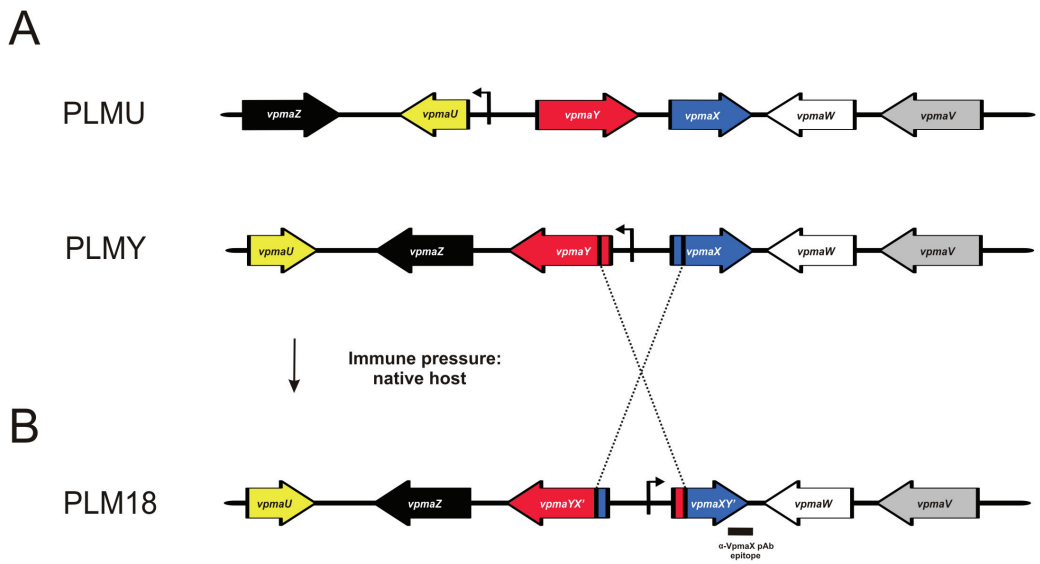
FIG. 7. (A) Restriction maps depicting the binding sites (black boxes) of *vpma*-specific gene probes within the *vpma* gene loci of PLMY, X-EV, PLM18 and PLM16: H, *Hind*III; P, *Pst*I; X, *Xba*I. (B) Southern blot analysis using *vpma*-specific probes: DIG-labeled DNA size marker (lane 1), PLMY carrying all six *vpma* genes was used as a positive control (lane 2), X-EV (lane 3), PLM18 (lane 4) and PLM16 (lane 5); *Hind*III/*Xba*I digested genomic DNA probed with *vpmaU*-specific probe detects

a 2.8 kb *Hind*III/*Xba*I fragment corresponding to the *vpmaU* gene in PLMY, X-EV and PLM18, which is absent in PLM16; *Hind*III/*Xba*I digested DNA probed with *vpmaV*-specific probe detects a 2.1 kb *Hind*III/*Xba*I fragment corresponding to the *vpmaV* gene present in all samples; *Hind*III digested DNA probed with *vpmaW*-specific probe detects a 2.8 kb *Hind*III fragment corresponding to the *vpmaW* gene in all samples, whereas an additional 2.6 kb *Hind*III fragment indicates duplication of *vpmaW* in PLM16; *Hind*III/*Xba*I digested DNA probed with *vpmaX*-specific probe verifies intergenic recombination between *vpmaX* and *vpmaY* in the X-EV: the *Hind*III site, originally present in the 5' region of *vpmaY* switched to the 5' end of the hybrid *vpmaYX* gene during recombination, the corresponing 0.5 kb *Hind*III fragment is detected after hybridization with a *vpmaX*-specific probe, a 1.7 kb *Hind*III fragment corresponds to *vpmaX* gene in PLMY and to the *vpmaX*-derived sequence of the hybrid *vpmaYX'* gene in PLM18, the presence of two *Hind*III/*Xba*I-fragments (0.8 kb and 1.9 kb) indicates duplication of *vpmaX* in PLM16; *Pst*I digested DNA probed with a *vpmaY*-specific probe detects a 0.6 kb *Pst*I fragment corresponding to the *vpmaY* gene in PLMY. In the *vpmaY*-derived sequence of the hybrid *vpmaXY* (X-EV) and *vpmaYX*' (PLM18) genes an additional 1.7 kb-*Pst*I fragment is observed corresponding to an adjacent untranscribed repeat of *vpmaY* and both these fragments are absent in PLM16; *Hind*III/*Xba*I digested DNA with *vpmaZ*-specific probe detects a 2.8 kb *Hind*III/*Xba*I fragment corresponding to the *vpmaZ* gene in PLMY, X-EV and PLM18, which is absent in PLM16. (C) Verification of *xer*1-disruption in X-EV (lane 3), PLM18 (lane 4), PLM16 (lane 5) using *Cla*I-digested genomic DNA and a *xer*1-specific probe was accomplished as described by Chopra-Dewasthaly et al. (2008); genomic DNA of PG2 (lane 1) and PLMY (lane 2) were used as negative and positive controls, DIG-labeled DNA size marker (lane 6).

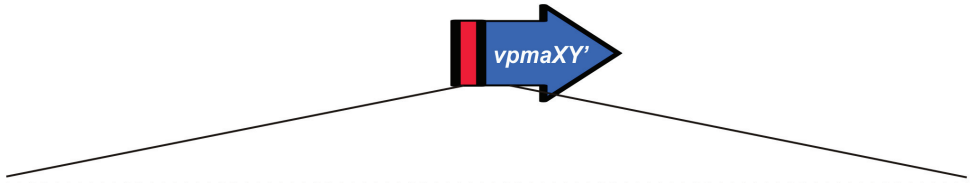
TABLE 1. Oligonucleotides

Oligonucleotide Name	Sequence (5' to 3')	Reference
1618P1	GCCCCATAATTAGAGCCC	This study
C1F	CCGGAATTCGTTGAGGAAGCAATTAAAACAGC	Chopra-Dewasthaly et al, 2008
C1R	GCTCTAGATTATCCAGATGTTTCAACTTC	Chopra-Dewasthaly et al, 2008
P2BPLMY	GGTGGTAGCACATCAACTG	This study
P2PLMU	TTTGAAGATGTGTTGGATG	This study
P6PLMU	CTCCAGCAGAAGGCAGTC	This study
P6PLMY	AGCGTTTGCATTGGCTAC	This study
P7PLMU	GTTACATTTACTGGAACTG	This study
P7PLMY	TTTTGTTGCCTAGTTTCTG	This study
P8PLMU	TAACGTCAGCAATGTCTG	This study
P8PLMY	TTATAGCCATTAAAGAAAGG	This study
P9PLMU	AGGCCTCATAATTATGAAG	This study
P9PLMY	AGTTTAGATTGACCTTTGG	This study
Prlocol1	CAAGTTATCAAGTTGCATGTAG	This study
PrSq1	AGCAAGGCAAAATTCAGAATATCTC	This study
PrSq2	AACTAAAACATTCTGTAATCTTAG	This study
TetMEnd	ACGCATAGTAGACCACCTC	This study
U2F	CGCGGATCCGATAAAGAAGATAAGACAGGTG	Chopra-Dewasthaly et al, 2008
Urev1	TCAACCTTAGATAAATCACCTAAC	This study
Urevvw4	GTTCTATCATGTCACTAGTTTG	This study
Vendrv	TAAATTTGAAGTATAAGTGA	This study
WDIGfw	CAACAATTAGGTGAAATCAAGAAG	This study
WDIGrv	AGAAGCATTTAAAGTGAATGAAACC	This study
Wstfw	TGATTTCTTTTCGTTTGTTG	This study
X1F	CGCGGATCCAAAGTAATGAAGGTCAATTACC	Chopra-Dewasthaly et al, 2008
X1R	AAACTGCAGGCTTAAGGATTTTTTAAAATGATG	Chopra-Dewasthaly et al, 2008
Y3F	CCGAATTCAATGCAAACGCTGCAGAAAATG	Chopra-Dewasthaly et al, 2008
Y3R	GCTCTAGATTAAGTAAATGTAACTGTAACTTCACC	Chopra-Dewasthaly et al, 2008
Yrevpr5	ATTAGTAGAAGATTGTAGCG	This study
Yrevv8	TTGTGCTTGCCTGTTACC	This study
Z1F	CGCGGATCCCAAACAGATTCAACTCCGTCAAC	Chopra-Dewasthaly et al, 2008
Z2R	AAACTGCAGTTATTCGTATTTAGGTAATAGTCTTC	Chopra-Dewasthaly et al, 2008
Zend1	AAGACTATTACCTAAATACG	This study

FIG. 1



C




```
          ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
vpmaXY'   ATGAAAAAATCAAAGTTTTTATTACTTGGATCAGTTGCTTCAATGGCCTCAATTCCTTTTGTGGCTGCTAAGTGTGGTGGTA (82)
vpmaX     ----------------------------------------------------------------------TGTGGTGGTA (82)
vpmaY     ATGAAAAAATCAAAGTTTTTATTACTTGGATCAGTTGCTTCAATGGCCTCAATTCCTTTTGTGGCTGCTAAGTGTGGTGGTA (82)

          ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
vpmaXY'   CTAAAGATGAAGAGAAGAAGCCAGCTGATACACAAGGTAGAGGTCAAAATAATCCCGGAGGAAATCAAAATCCAGGAACTGA (164)
vpmaX     CTAAAGATGAAGAGAAGAAGCCAGCTGATACACAAGGTAGAGGTCAAAATAATCCCGGAGGAAATCAAAATCCAGGAACTGA (164)
vpmaY     CTAAAGATGAAGAGAAGAAGCCAGCTGA---------------------------------------------------- (164)
```

FIG. 2

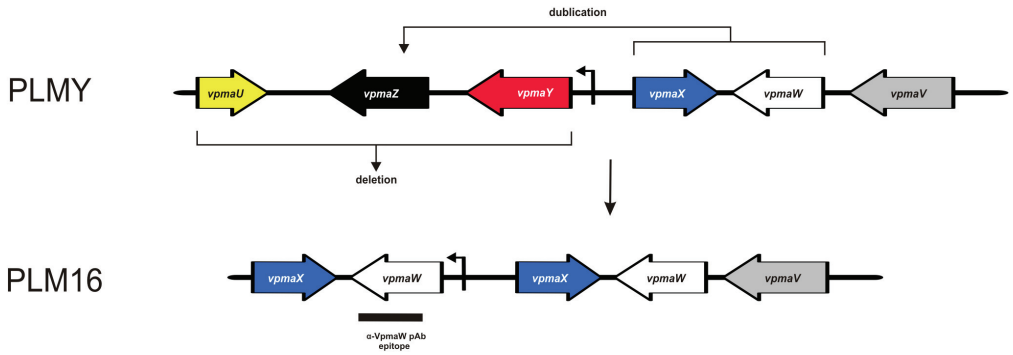

dublication
PLMY
vpmaU
vpmaZ
vpmaY
vpmaX
vpmaW
vpmaV
deletion
PLM16
vpmaX
vpmaW
vpmaX
vpmaW
vpmaV
α-VpmaW pAb epitope

FIG. 3

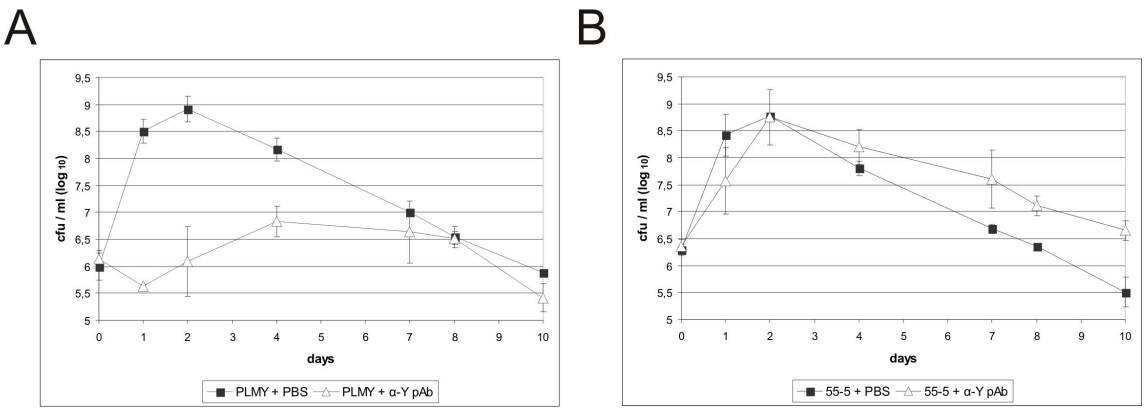


FIG. 4

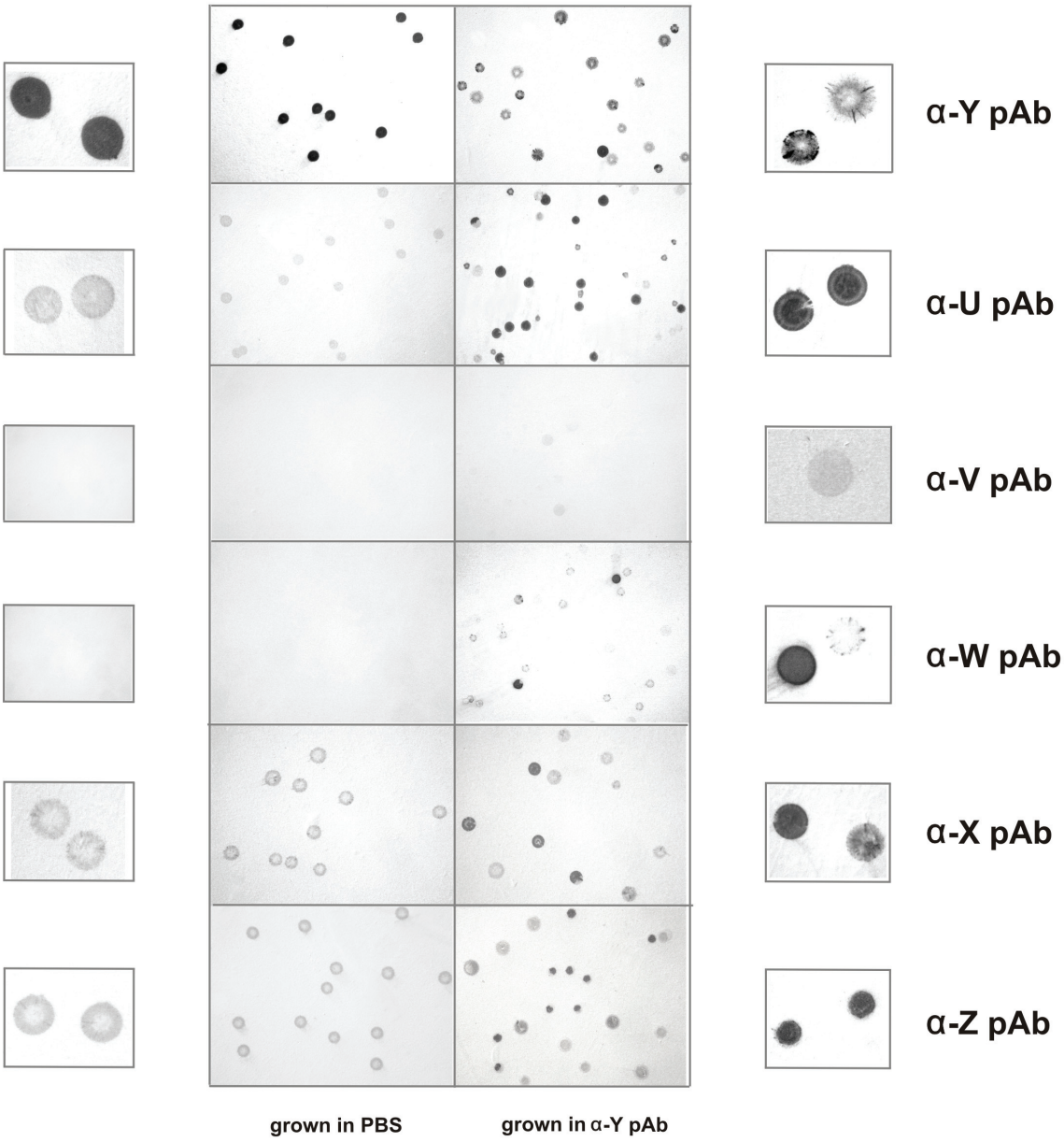

α-Y pAb
α-U pAb
α-V pAb
α-W pAb
α-X pAb
α-Z pAb
grown in PBS
grown in α-Y pAb

FIG. 5

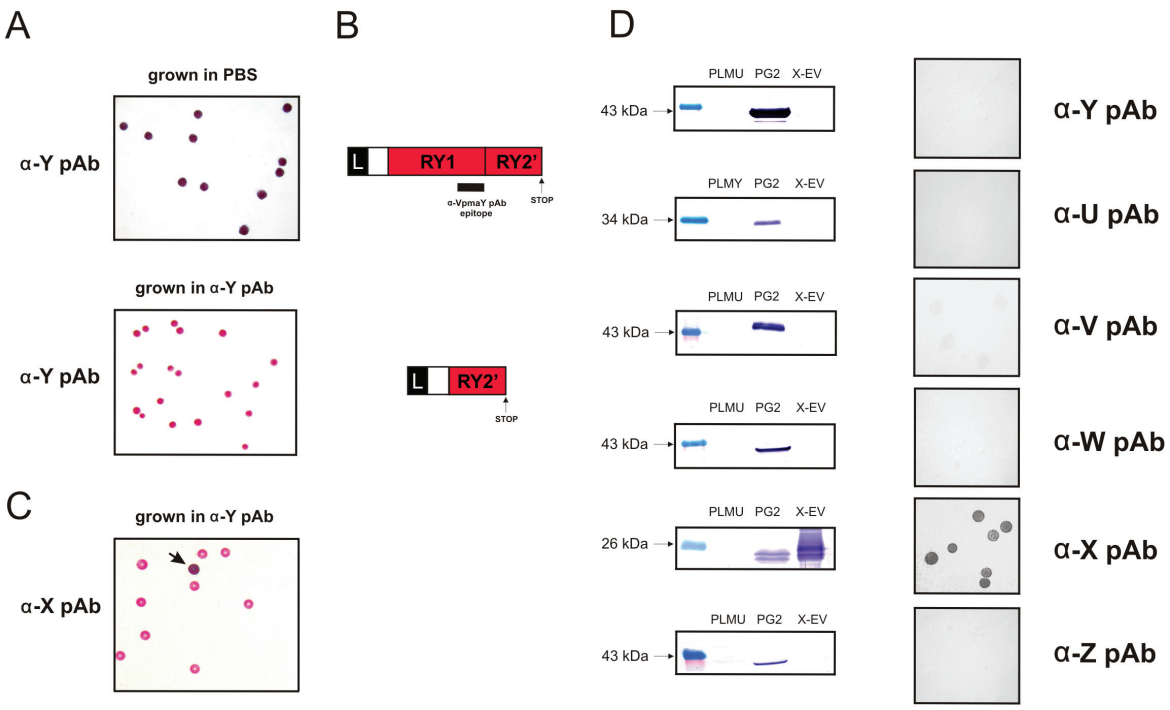
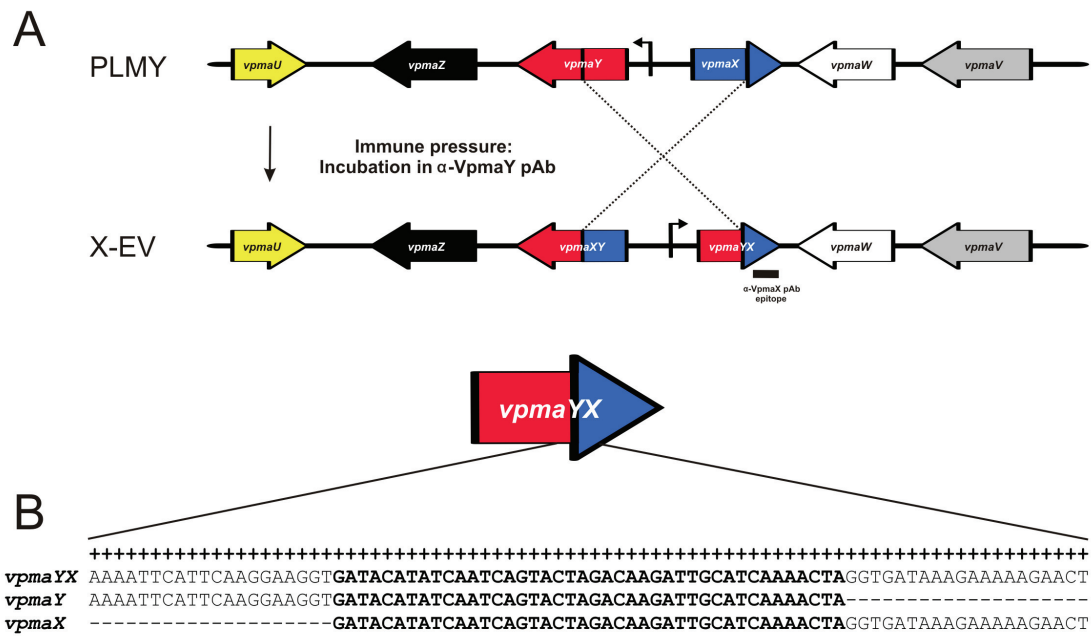
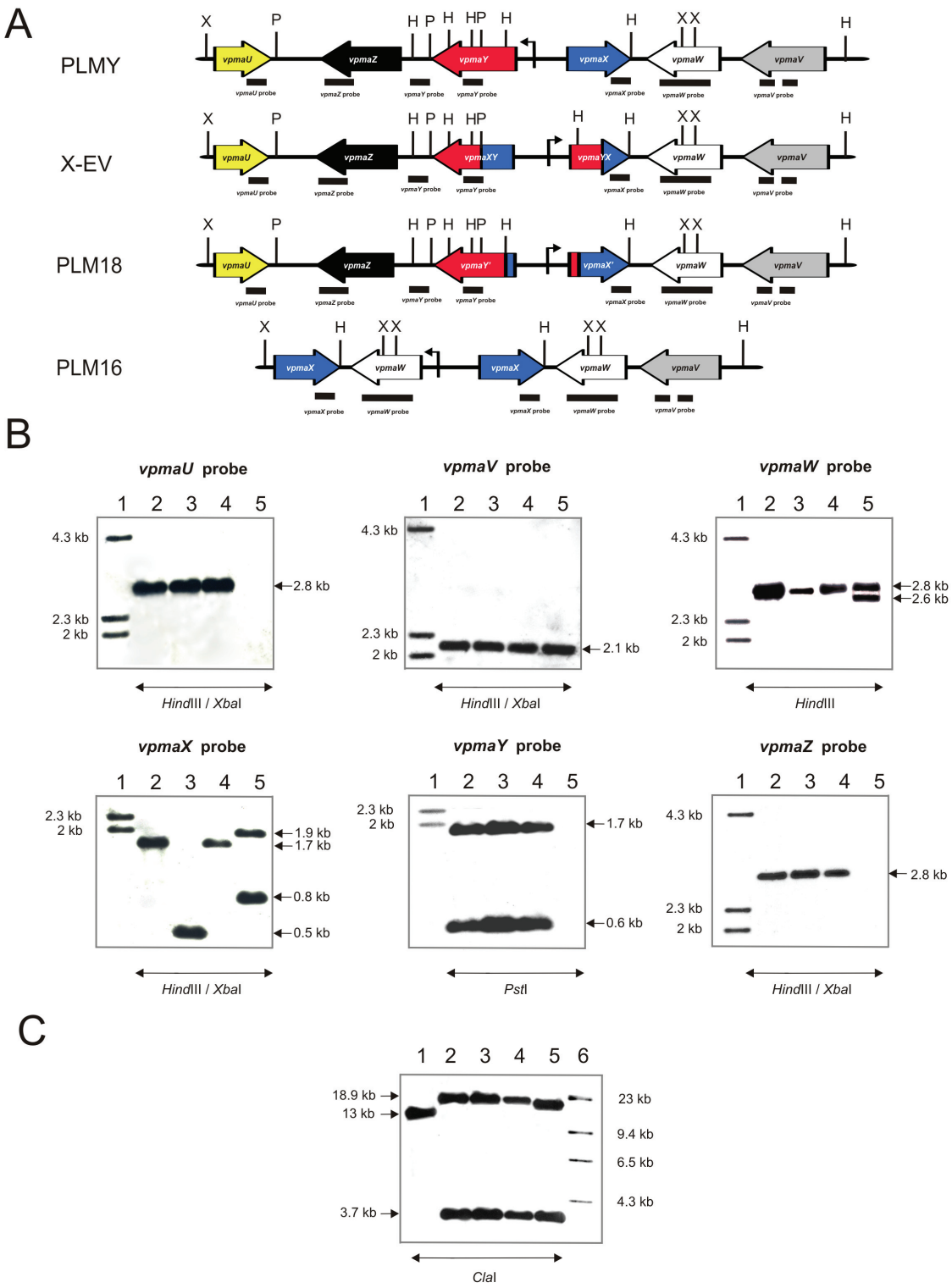

A
grown in PBS
α-Y pAb
grown in α-Y pAb
α-Y pAb
B
L
RY1
RY2'
α-VpmaY pAb epitope
STOP
L
RY2'
STOP
C
grown in α-Y pAb
α-X pAb
D
PLMU PG2 X-EV
43 kDa
PLMY PG2 X-EV
34 kDa
PLMU PG2 X-EV
43 kDa
PLMU PG2 X-EV
43 kDa
PLMU PG2 X-EV
26 kDa
PLMU PG2 X-EV
43 kDa
α-Y pAb
α-U pAb
α-V pAb
α-W pAb
α-X pAb
α-Z pAb

FIG. 6




```
++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
vpmaYX AAAATTCATTCAAGGAAGGTGATACATATCAATCAGTACTAGACAAGATTGCATCAAAACTAGGTGATAAAGAAAAAGAACT (82)
vpmaY  AAAATTCATTCAAGGAAGGTGATACATATCAATCAGTACTAGACAAGATTGCATCAAAACTA------------------- (82)
vpmaX  --------------------GATACATATCAATCAGTACTAGACAAGATTGCATCAAAACTAGGTGATAAAGAAAAAGAACT (82)
```

FIG. 7


A
PLMY
X-EV
PLM18
PLM16
vpmaU
vpmaZ
vpmaY
vpmaX
vpmaW
vpmaV
vpmaXY
vpmaYX
vpmaY'
vpmaX'
B
vpmaU probe
vpmaV probe
vpmaW probe
vpmaX probe
vpmaY probe
vpmaZ probe
1 2 3 4 5
4.3 kb
2.3 kb
2 kb
2.8 kb
2.1 kb
2.6 kb
1.9 kb
1.7 kb
0.8 kb
0.5 kb
0.6 kb
HindIII / XbaI
HindIII
PstI
C
1 2 3 4 5 6
18.9 kb
13 kb
3.7 kb
23 kb
9.4 kb
6.5 kb
4.3 kb
ClaI

6 Appendix

6.1 Solutions and Media

6.1.1 Media, media additives and antibiotics

SP4 Broth	0.35% mycoplasma broth base (Oxoid) 1% tryptone (Oxoid) 0.53% peptone (Merck) pH 7.8 with NaOH (Sigma) autoclaved at 120°C for 20 min 17 ml/100 ml heat inactivated fetal calf serum (Gibco) 5 ml/100 ml CMRL (Invitrogen) 5 ml/100 ml 4% aqueous yeastolate solution (Difco) 2.5 ml/100 ml 15% filter sterile yeast extract (Merck) solution 1 ml/100 ml 50% filter sterile glucose (Roth) solution 1 ml/100 ml 0.5% filter sterile phenol red solution (ICN Biomedicals) 2 ml/100 ml 25% filter sterile sodium pyruvate (Sigma) solution
SP4 Agar Plates	SP4 broth with 1% noble agar (Difco) before autoclaving ddH_2O instead of phenol red and sodium pyruvate solutions
LB Broth	5% yeast extract (Merck) 10% NaCl (Roth) 10% tryptone (Oxoid)
LB Agar Plates	LB broth with 1.5% agar (Oxoid)
Ampicillin Stock (Roth)	100 mg/ml in ddH_2O
Tetratcycline Stock (Sigma)	10 mg/ml in ddH_2O
Gentamicin Stock (Roth)	10 mg/ml in ddH_2O

Glucose Stock (Roth)	50% in ddH_2O, filter sterile
Arabinose Stock (Roth)	10% in ddH_2O, filter sterile

6.1.2 Southern blot analysis

Depurination Solution	250 mM NaCl (Roth)
Denaturation Solution	500 mM NaOH (Roth) 1.5 M NaCl (Roth)
Neutralization Solution	500 mM Tris (Roth) 3 M NaCl (Roth) pH 7.5 adjusted with HCl (Roth)
20 x Sodium Chloride / Sodium Citrate Buffer (SSC)	3 M NaCl (Roth) 300 mM sodium citrate (Roth)
Maleic Acid Buffer	100 mM maleic acid (Roth) 150 mM NaCl (Roth) pH 7.5 adjusted with NaOH (Roth)
Washing Solution	maleic acid buffer 0.3% Tween 20 (Roth)
Blocking Solution	maleic acid buffer 1% blocking reagent (Roche)
Detection Buffer	100 mM Tris (Roth) 100 mM NaCl (Roth) pH 9.5 adjusted with NaOH (Roth)

6.1.3 Agarose gel electrophoresis

6 x DNA Loading Buffer	40% glycerol (Roth) 0.1% bromophenol blue (Sigma)
50 x TAE	2 M Tris (Roth) 1 M acetic acid (Roth) 500 mM EDTA (Merck)
Agarose Gel	0.8 - 1% agarose (Roth) boiled in 1 x TAE

6.1.4 DNA isolation

Washing Solution	250 mM NaCl (Roth) 100 mM EDTA (Merck)
Suspension Solution	50 mM Tris pH 8.0 (Roth) 10 mM EDTA (Merck)
Phenol / Chloroform / Isoamyl Alcohol Solution (25:24:1)	(Sigma)
TE Buffer	10 mM Tris pH 8.0 (Roth) 1 mM EDTA (Merck)
RNase Stock (Roth)	10 mg/ml in ddH_2O
Proteinase K Stock (Roth)	10 mg/ml in 10 mM Tris pH 8.0 (Roth)
Potassium Acetate	5 M potassium acetate (Roth) in ddH_2O
Sodium Acetate	3 M sodium acetate (Roth) pH 5.2 adjusted with HCl (Roth)

6.1.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

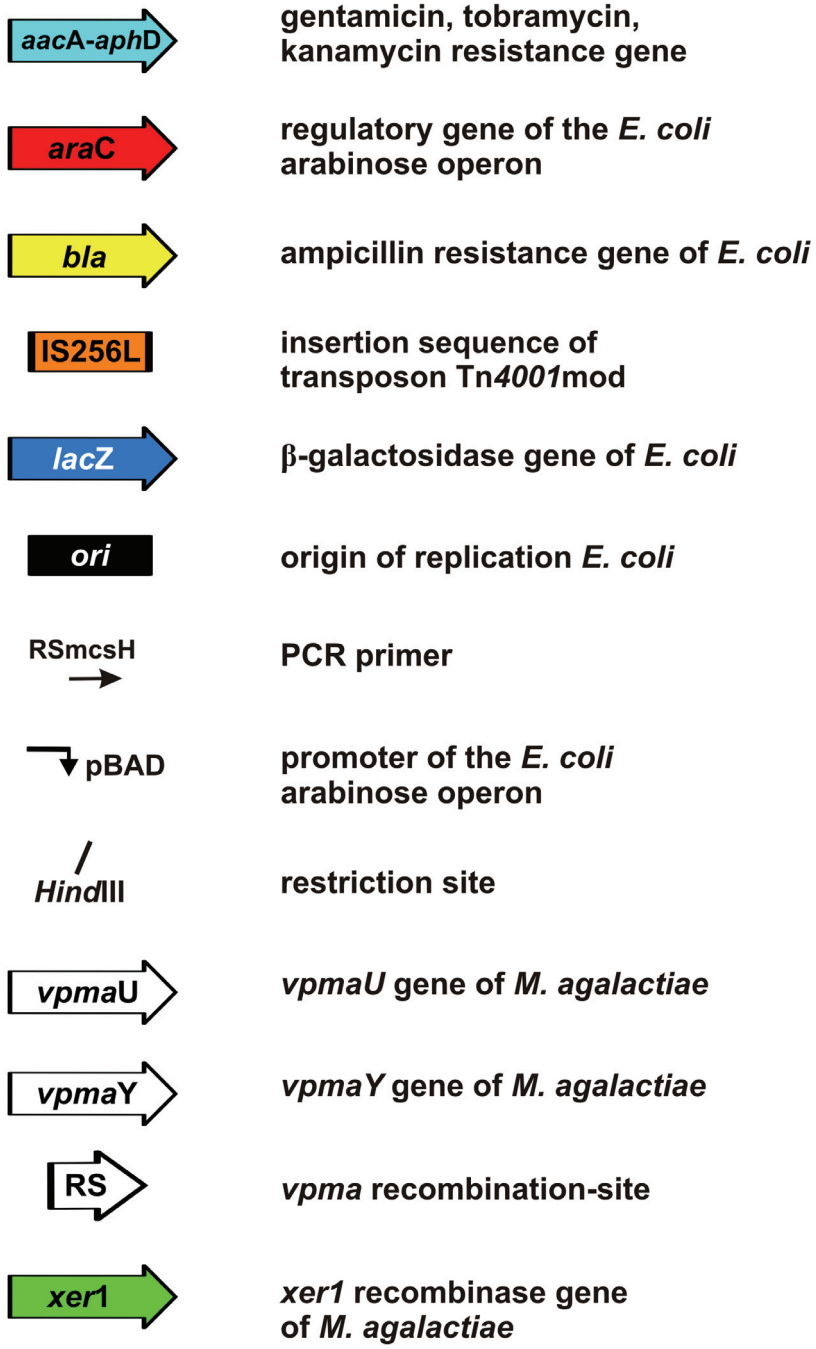
APS Solution	10% ammonium peroxide sulfate (Roth) in ddH_2O
40% Acrylamid Solution	(Roth)
Urea Stock	30% urea (Roth) in ddH_2O
SDS Stock	10% SDS (Roth) in ddH_2O
TEMED Solution	(Roth)
Resolving Gel Buffer	1.5 M Tris (Roth) pH 8.8 adjusted with HCl (Roth)
Stacking Gel Buffer	1 M Tris (Roth) pH 6.8 adjusted with HCl (Roth)
5 x Loading Buffer	250 mM Tris-HCl pH 6.8 (Roth) 50% glycerol (Roth) 10% SDS (Roth) 7.5% DTT (Roth) 0.1% bromophenol blue (Sigma)
10 x Laemmli Electrode Buffer (LEB)	250 mM Tris (Roth) 1.9 M glycine (Roth)

6.1.6 Western blot and colony blot analysis

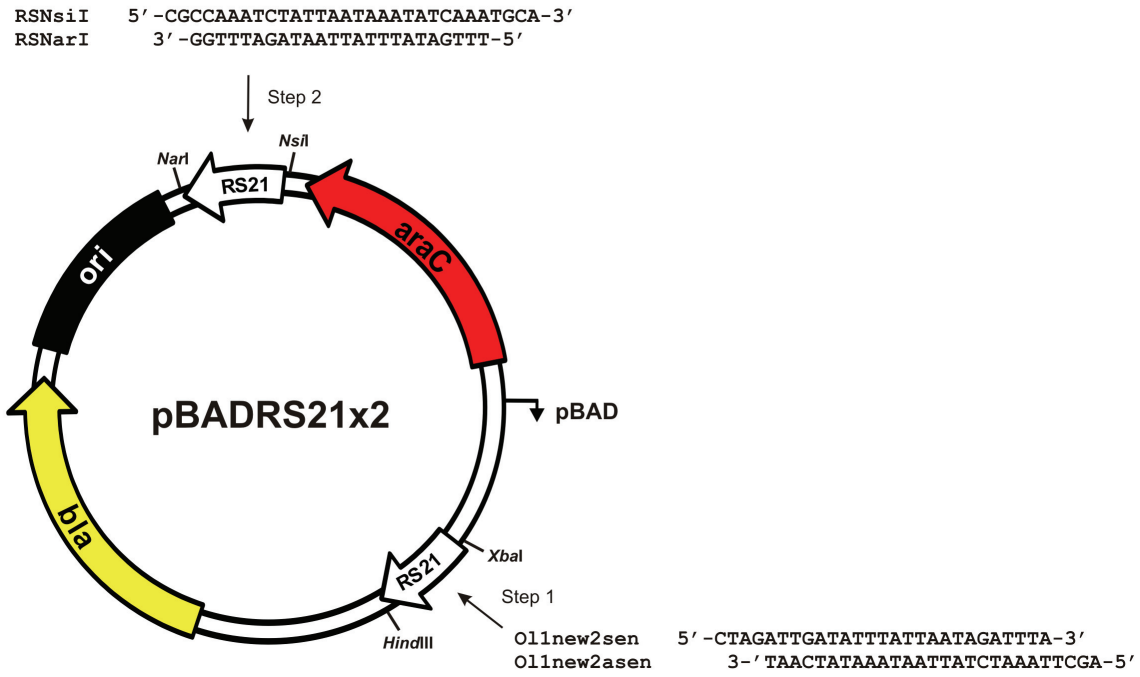
10 x Tris Buffered Saline (TBS)	1.5 M NaCl (Roth) 100 mM Tris (Roth) pH 7.4 adjusted with HCl (Roth)
TBS Tween	1 x TBS 0.05% Tween 20 (Roth)
Ponceau Stain	0.25% Ponceau S (Roth) 5% acetic acid (Roth)
Blocking Solution	3% milkpowder (Bio-Rad) in 1x TBS
CN Stock	0.3% 1-chloro-4-naphthol (Bio-Rad) in 100% methanol (Roth)
CN Working Solution	2 ml CN stock 10 ml 10 x TBS 10 µl H_2O_2
Blotting Buffer	1 x LEB 20% methanol (Roth)

6.2 Plasmid constructs

6.2.1 Symbols and abbreviations



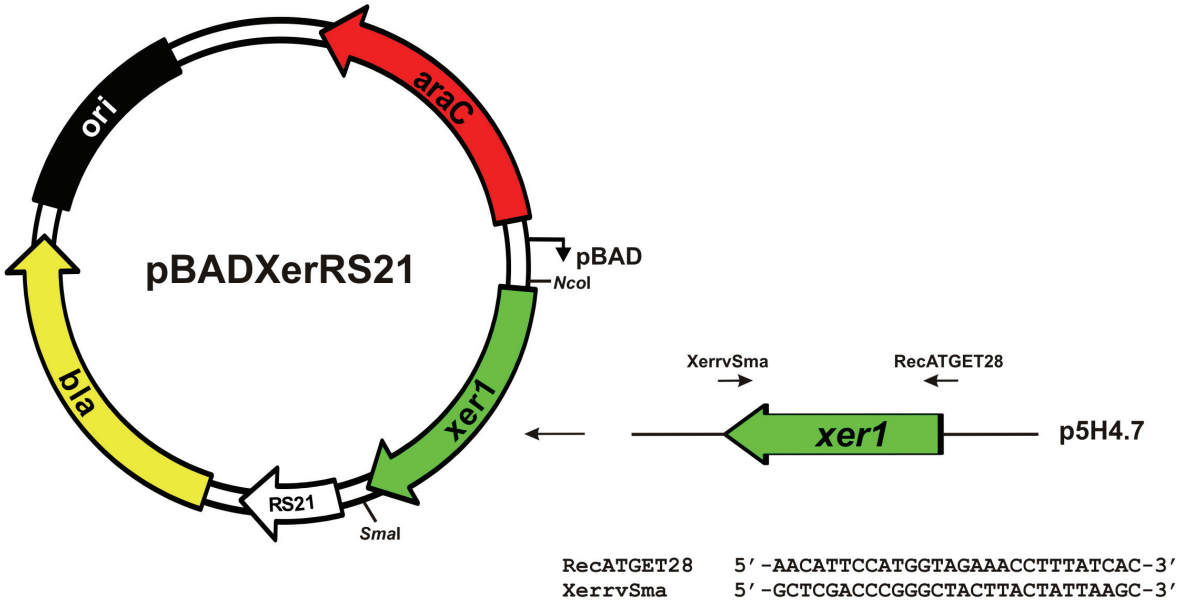
6.2.2 Construction of plasmid pBADRS21x2



Step 1: RS21 was cloned into *Hind*III and *Xba*I sites of pBAD24 (Guzman et al., 1995) leading to plasmid pBADRS21

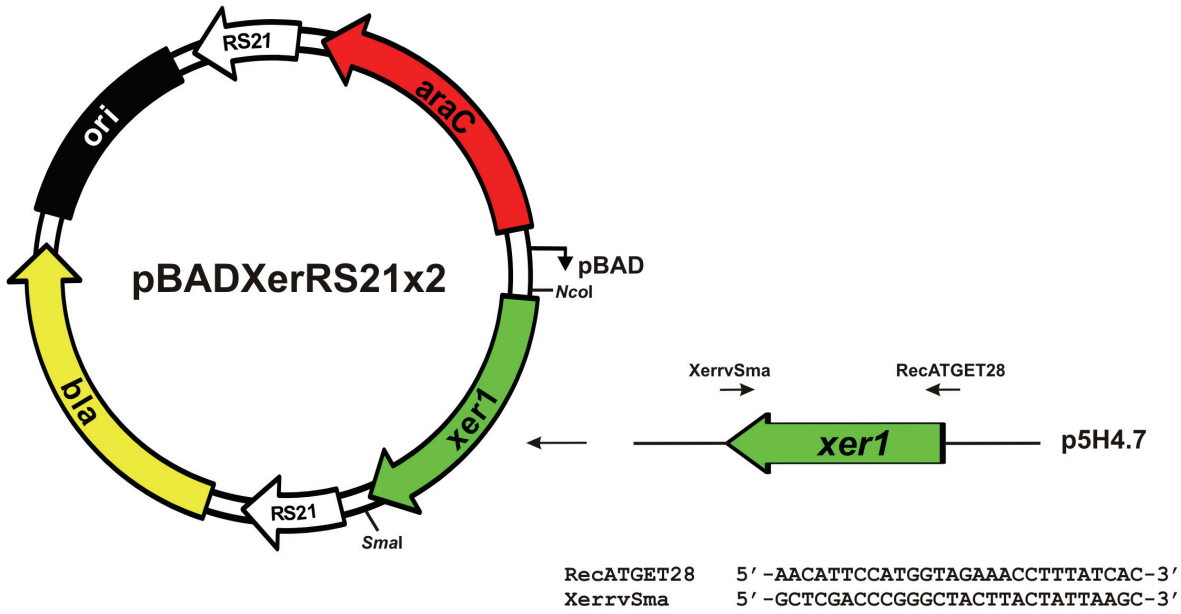
Step 2: RS21 was cloned into *Nar*I and *Nsi*I sites of pBADRS21 leading to plasmid pBADRS21x2

6.2.3 Construction of plasmid pBADXerRS21



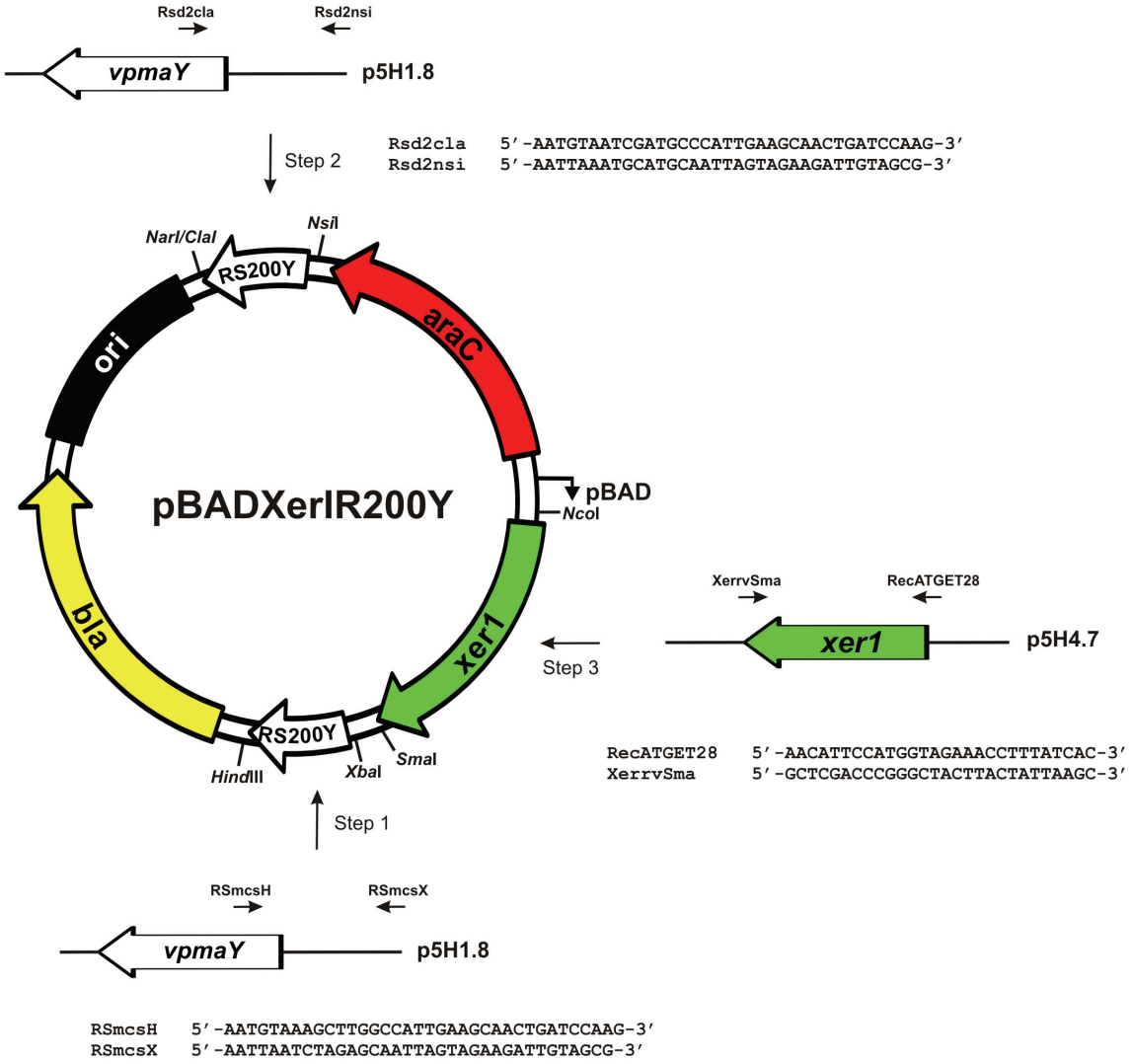
xer1 was cloned into *Nco*I and *Sma*I sites of pBADRS21 leading to plasmid pBADXerRS21

6.2.4 Construction of plasmid pBADXerRS21x2



xer1 was cloned into *Nco*I and *Sma*I sites of pBADRS21x2 leading to plasmid pBADXerRS21x2

6.2.5 Construction of plasmid pBADXerIR200Y

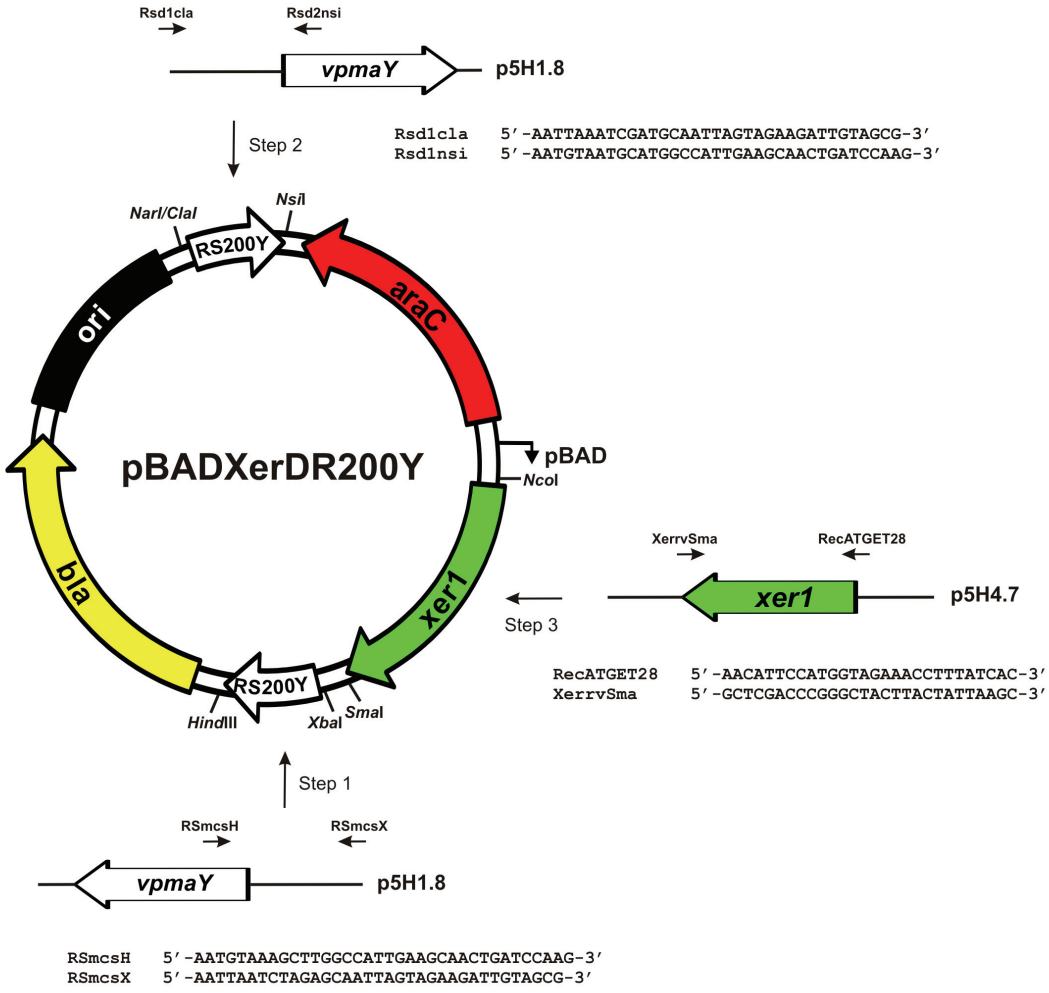


Step 1: RS200Y was cloned into *Hind*III and *Xba*I sites of pBAD24 leading to plasmid pBADRS

Step 2: RS200Y was cloned into *Nar*I/*Cla*I and *Nsi*I sites of pBADRS leading to plasmid pBADRSIR

Step 3: *xer1* was cloned into *Nco*I and *Sma*I sites of pBADRS21x2 leading to plasmid pBADXerIR200Y

6.2.6 Construction of plasmid pBADXerDR200Y

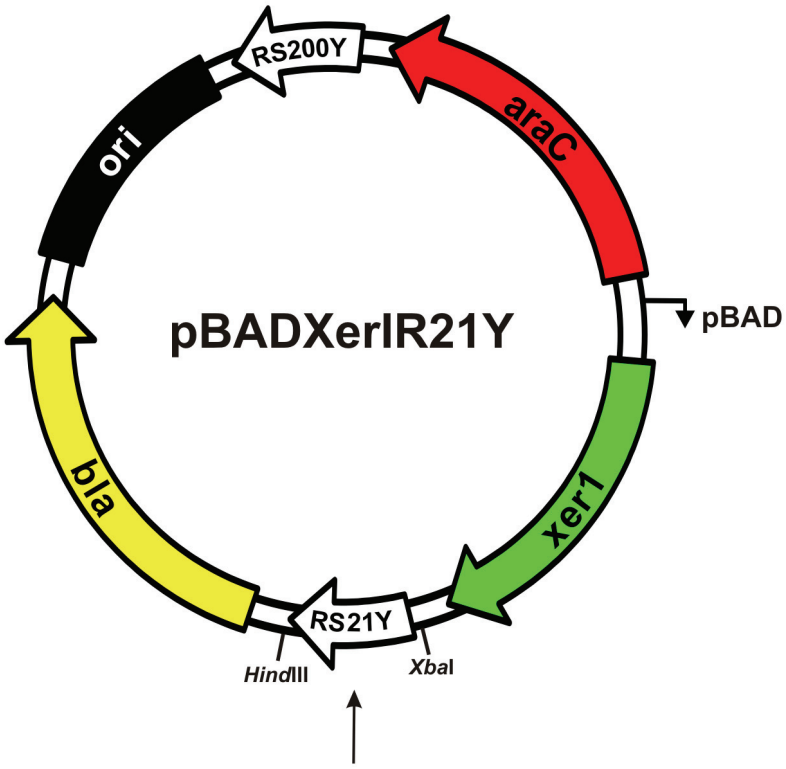


Step 1: RS200Y was cloned into *Hind*III and *Xba*I sites of pBAD24 leading to plasmid pBADRS

Step 2: RS200Y was cloned into *Nar*I/*Cla*I and *Nsi*I sites of pBADRS leading to plasmid pBADRSDR

Step 3: *xer1* was cloned into *Nco*I and *Sma*I sites of pBADRS21x2 leading to plasmid pBADXerDR200Y

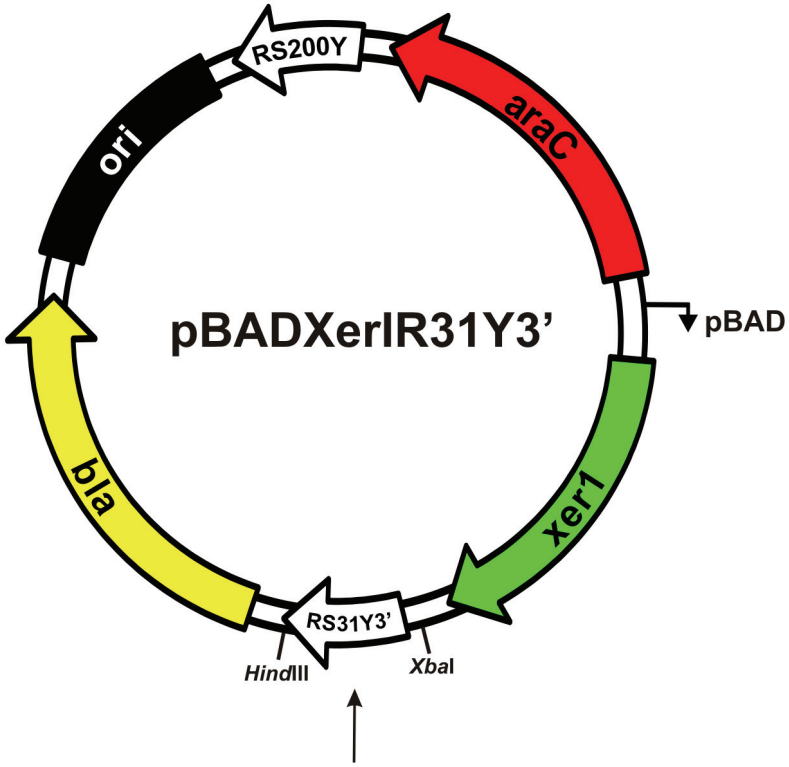
6.2.7 Construction of plasmid pBADXerIR21Y



Oll new2sen 5′-CTAGATTGATATTTATTAATAGATTTA-3′
Oll new2asen 3′-TAACTATAAATAATTATCTAAATTCGA-5′

RS21Y was cloned into *Hind*III and *Xba*I digested pBADXerIR200Y to replace RS200Y leading to plasmid pBADXerIR21Y

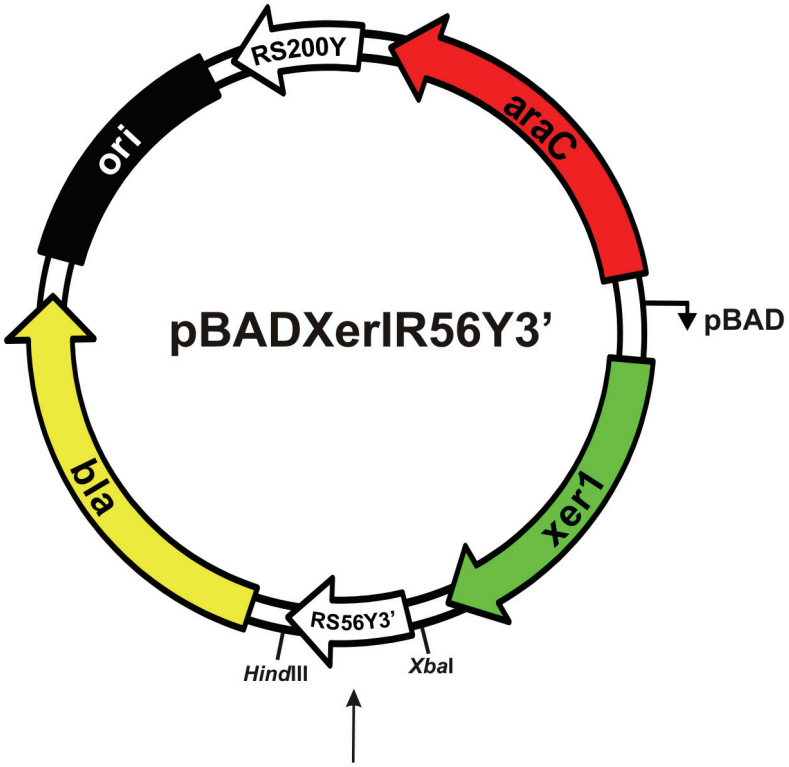
6.2.8 Construction of plasmid pBADXerIR31Y3’



Oligo#2sense 5′-CTAGTTGATATTTATTAATAGATTTATAAAGCATTA-3′
Oligo#2asense 3′-AACTATAAATAATTATCTAAATATTTCGTAATTCGA-5′

RS31Y3’ was cloned into *Hind*III and *Xba*I digested pBADXerIR200Y to replace RS200Y leading to plasmid pBADXerIR31Y3’

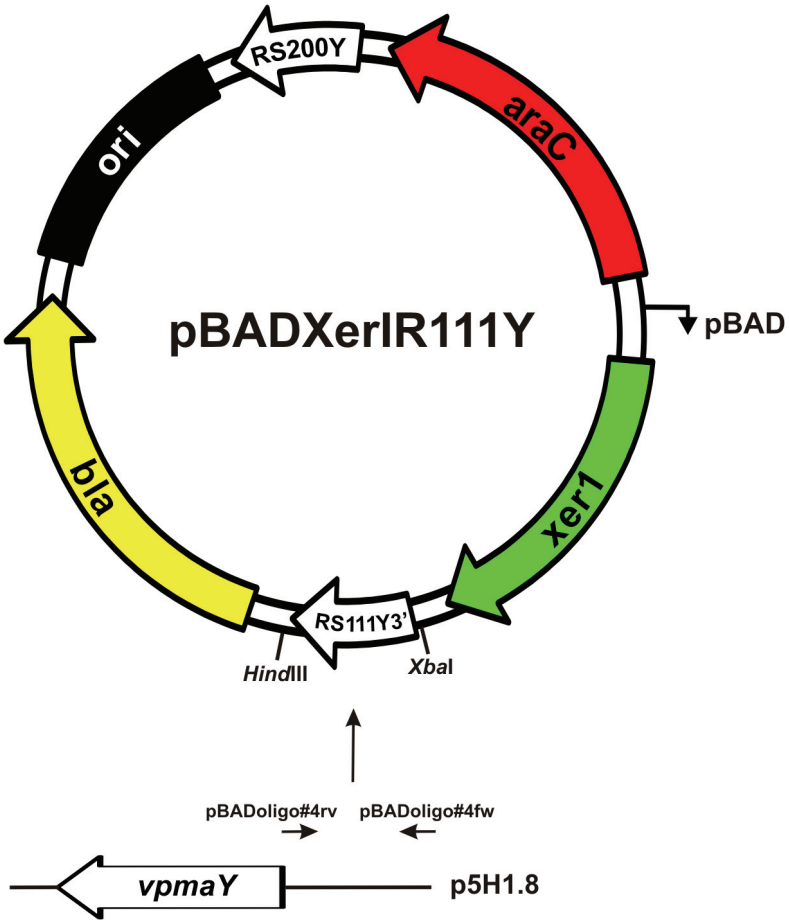
6.2.9 Construction of plasmid pBADXerIR56Y3’




```
Oligo#3sense     5′-CTAGTTGATATTTATTAATAGATTTATAAAGCATTA-3′
Oligo#3asense       3′-AACTATAAATAATTATCTAAATATTTCGTAATTCGA-5′
```

RS56Y3’ was cloned into *Hind*III and *Xba*I digested pBADXerIR200Y to replace RS200Y leading to plasmid pBADXerIR56Y3’

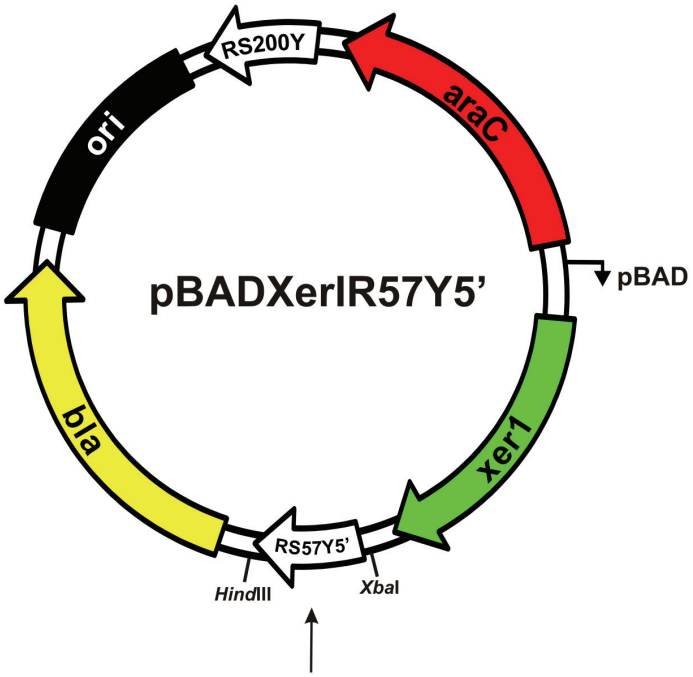
6.2.10 Construction of plasmid pBADXerIR111Y



pBADoligo#4fw 5′-GATCAATCTAGAGCTATTAATTTAGCACTTAATAC-3′
pBADoligo#4rv 5′-AATGTCAAGCTTTCATAAATTTATCCTTTC-3′

RS111Y was cloned into *Hind*III and *Xba*I digested pBADXerIR200Y to replace RS200Y leading to plasmid pBADXerIR111Y

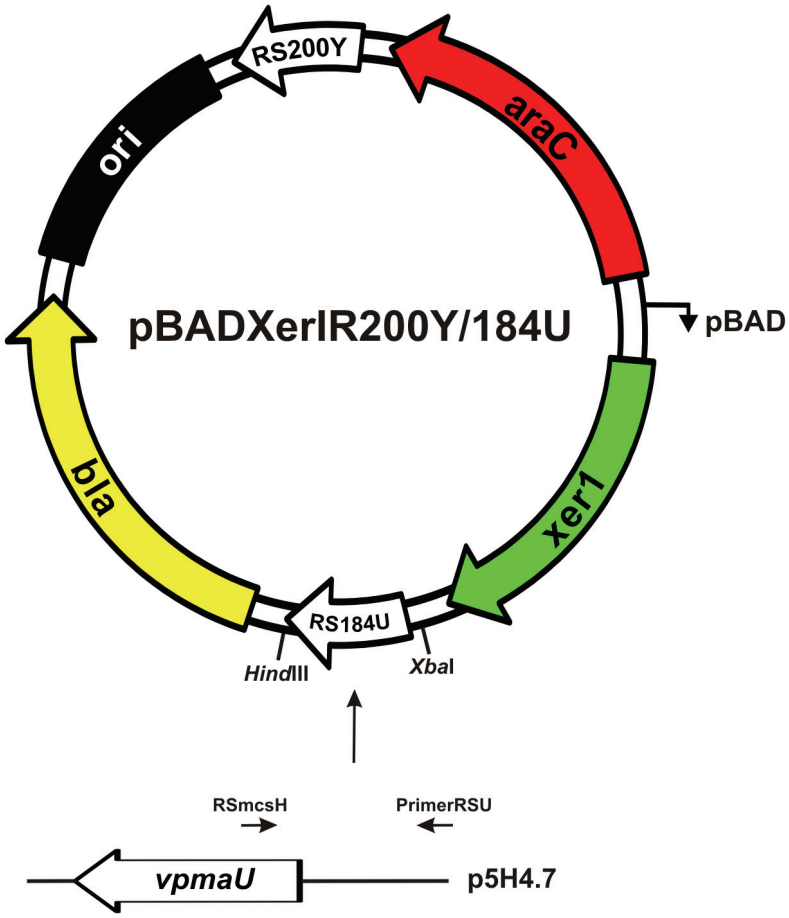
6.2.11 Construction of plasmid pBADXerIR57Y5’




```
Ol9sen    5’-CTAGAGCTATTAATTTAGCACTTAATACATCATATAATAAATTGATATTATTAATAGATTTA-3’
Ol9asen      3’- TCGATAATTAAATCGTGAATTATGTAGTATATTATTTAACTATAATAATTATCTAAATTCGA-5’
```

RS57Y5’ was cloned into *Hind*III and *Xba*I digested pBADXerIR200Y to replace RS200Y leading to plasmid pBADXerIR57Y5’

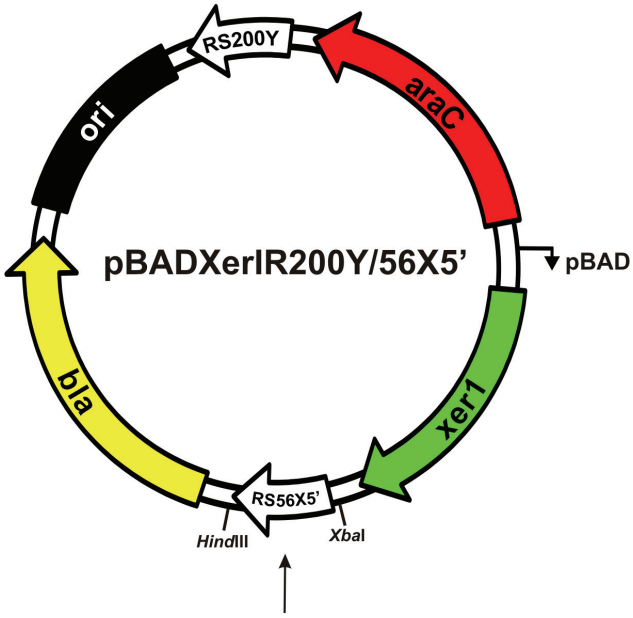
6.2.12 Construction of plasmid pBADXerIR200Y/184U



RSmcsH 5′-AATGTAAAGCTTGGCCATTGAAGCAACTGATCCAAG-3′
PrimerRSU 5′-AATTGCTCTAGAAAGTTTAATCAAAACTTAACG-3′

RS184U was cloned into *Hind*III and *Xba*I digested pBADXerIR200Y to replace RS200Y leading to plasmid pBADXerIR200Y/184U

6.2.13 Construction of plasmid pBADXerIR200Y/56X5’

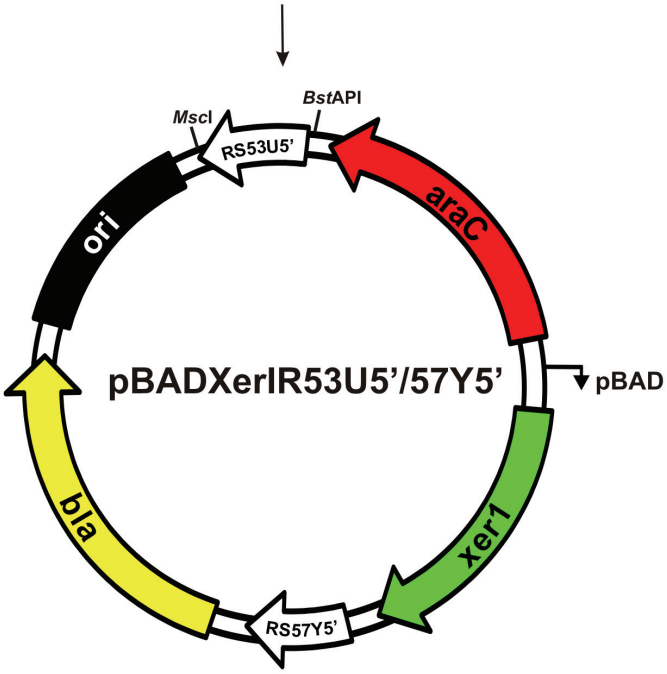



```
OligoRSXsen     5′-CTAGATTTAGTAAGCAGTCGCTAAGCATATACTTGTACTTTTGATATTTATTAATAGATTTA-3′
OligoRSXasen        3′-TAAATCATTCGTCAGCGATTCGTATATGAACATGAAAACTATAAATAATTATCTAAATTCGA-5′
```

RS56X5’ was cloned into *Hind*III and *Xba*I digested pBADXerIR200Y to replace RS200Y leading to plasmid pBADXerIR200Y/56X5’

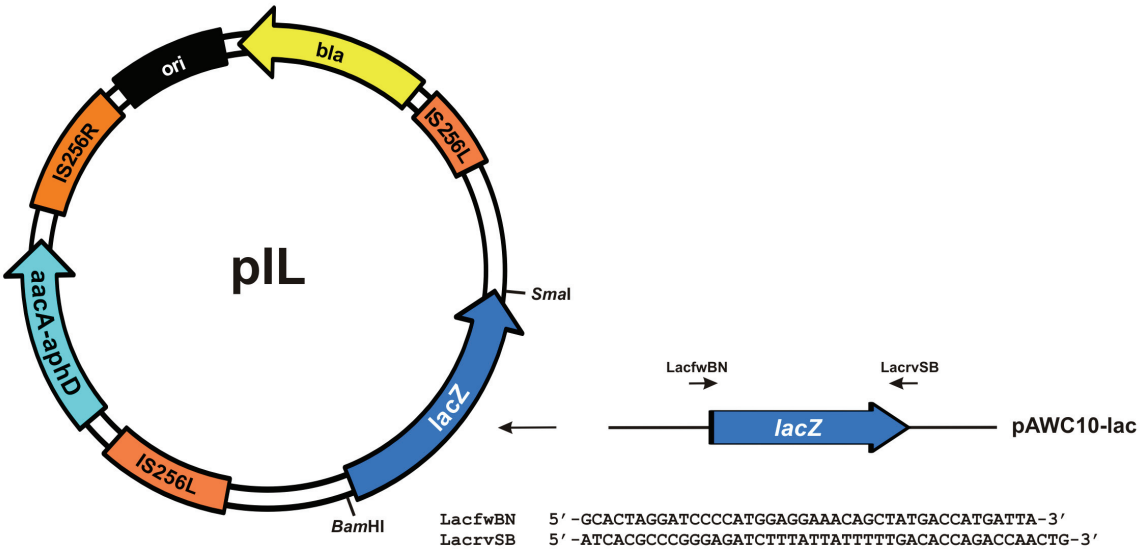
6.2.14 Construction of plasmid pBADXerIR53U5'/57Y5'

```
OligoRSUsen          5′-TGATGTTTTTATATTCTCATAAACTCACTGTTTTTGATATTTATTAATAGATTTTGG-3′
OligoRSUasen    5′-ACGTACTACAAAAATATAAGAGTATTTGAGTGACAAAAACTATAAATAATTATCTAAAACC-5′
```



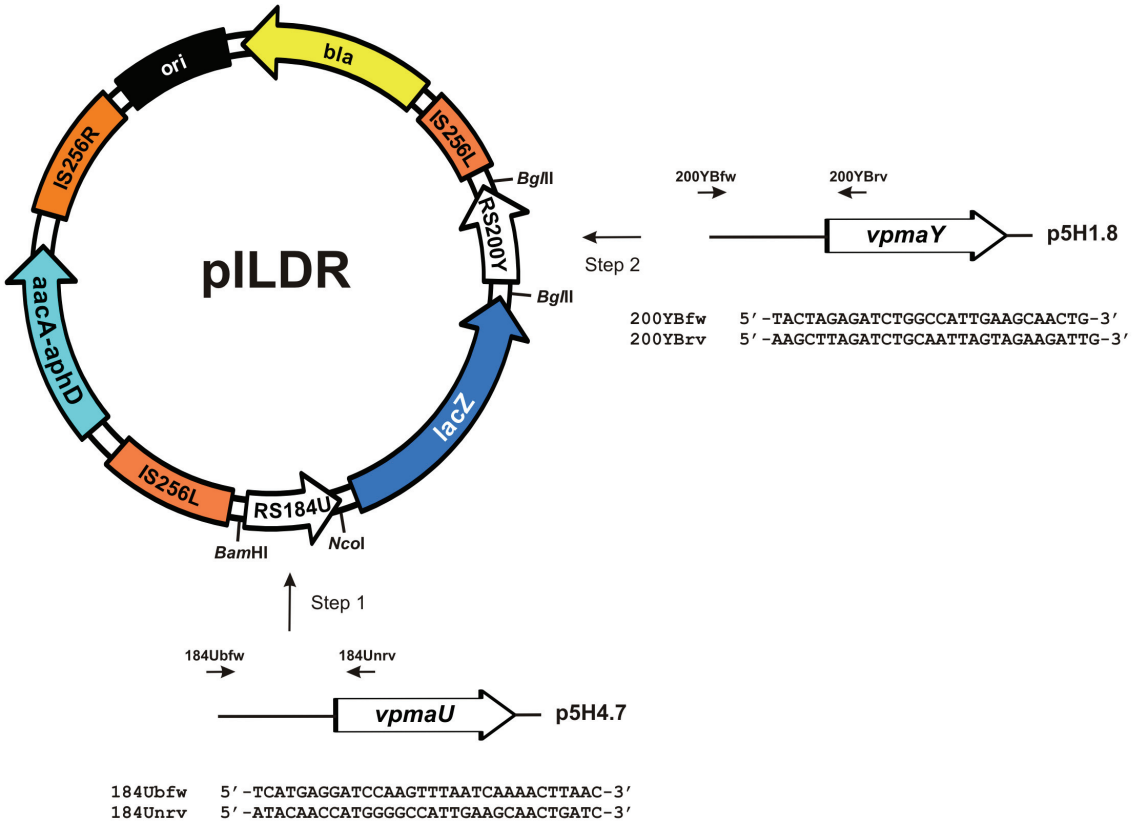
RS53U5' was cloned into *Msc*I and *Bst*API digested pBADXerIR57Y5' to replace RS200Y leading to plasmid pBADXerIR53U5'/57Y5'

6.2.15 Construction of plasmid pIL



lacZ was cloned into *Bam*HI and *Sma*I sites of pISM2062 (Knudtson and Minion, 1993) leading to plasmid pIL

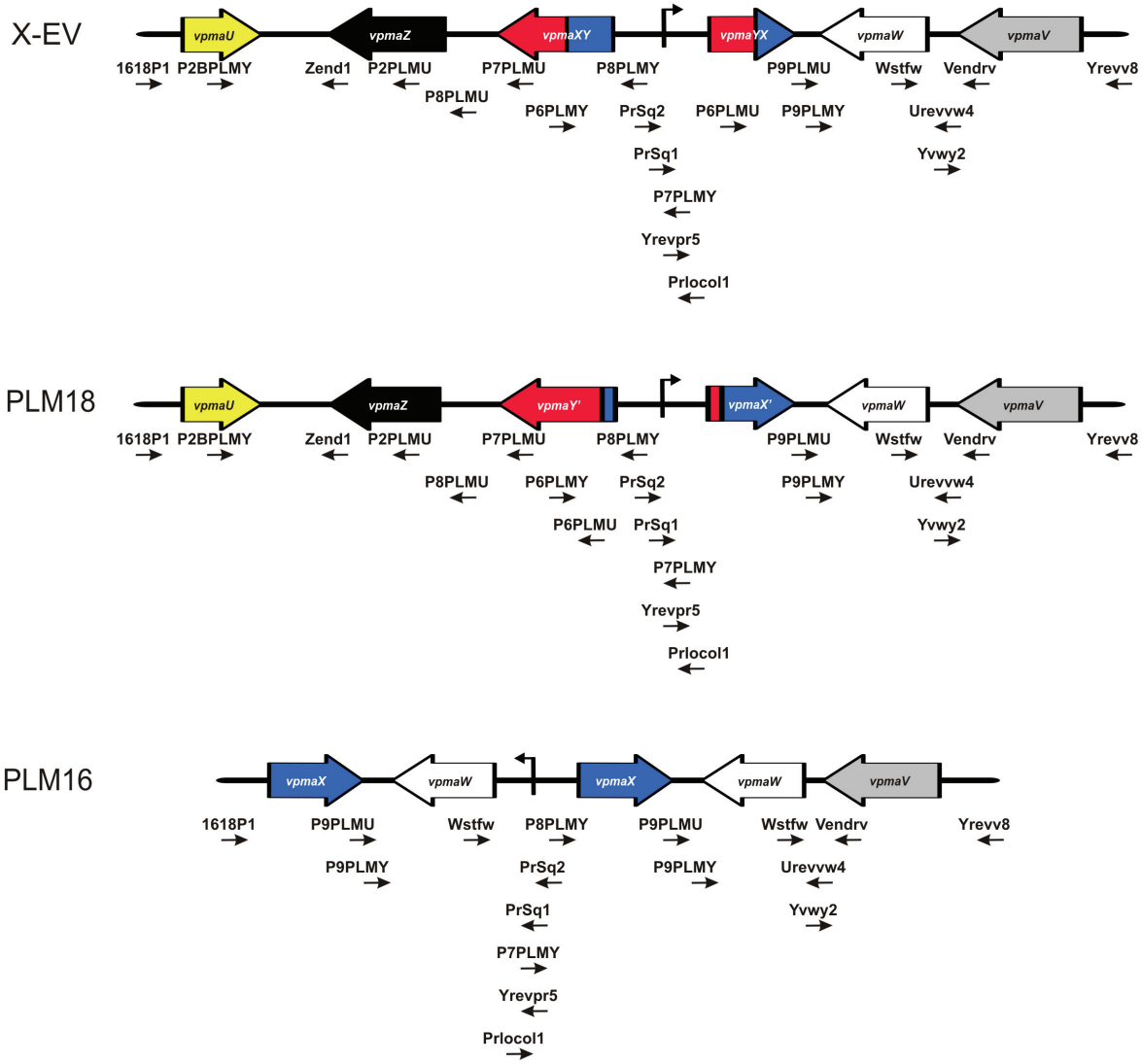
6.2.16 Construction of plasmid pILDR



Step 1: RS184U was cloned into *Bam*HI and *Nco*I sites of pIL leading to plasmid pILRSU

Step 2: RS200Y was cloned into *Bgl*II site of pILRSU leading to plasmid pILDR

6.3 *vpma* locus of X-EV, PLM18 and PLM16 – annealing sites of sequencing primers



Acknowledgements

Ich möchte mich ganz herzlich bedanken…

bei meiner Dissertationsbetreuerin Prof. Dr. Dr. habil. Renate Rosengarten, die mir die Möglichkeit gab, meine Dissertationsarbeit am Institut für Bakteriologie, Mykologie und Hygiene der Veterinärmedizinischen Universität Wien durchzuführen. Zusätzlich möchte ich mich bedanken, dass ich bei so vielen nationalen und internationalen Kongressen aktiv teilnehmen durfte, die für mich eine sehr wertvolle Erfahrung waren.

bei der Arbeitsgruppenleiterin innerhalb der Mykoplasmen-Forschungsgruppe Dr. Rohini Chopra-Dewasthaly, die mich über fast sechs Jahre im Labor begleitet und betreut hat. Aufgrund ihrer langjährigen Erfahrungen durfte ich viel von ihr lernen. Vielen Dank für unser freundschaftliches Arbeitsverhältnis und für die nette Unterstützung bei der Labortätigkeit und beim Erstellen meiner Dissertation und meines Manuskriptes.

bei Prof. Dr. Angela Witte für ihre nette administrative Unterstützung in Studienangelegenheiten und für ihre wertvollen fachlichen Anregungen beim Erstellen meines Manuskriptes.

bei Dr. Micheal Szostak für seine hilfreiche Diskussion in fachlichen Fragen und für das Lösen von Computerproblemen

bei den technischen Assistenten Mag. Martina Zimmermann und Karin Siebert-Gulle, ohne die ich im Labor sehr oft verloren gewesen wäre.

bei meinen Kollegen Dr. Gunther Vogl, Dr. Ivana Indikova, Mag. Martin Vronka, Mag. Shivanand Hegde, Dr. Ursula Fürnkranz, Mag. Isolde Nieszner und Dr. Carl-Ulrich Zimmerman, die mir bei der täglichen Labortätigkeit eine große Hilfe waren.

bei Isabell Polonyi, Helga Brödl und Christine Schramm für ihre nette administrative Unterstützung.

bei meiner Freundin Cathrien Egger für ihre seelische Unterstützung in Zeiten, wo es nicht so gut lief.

bei meinen Eltern Erhard and Erika Czurda für die langjährige finanzielle Unterstützung während meines Studiums.

Curriculum Vitae

Personal Data

Date of birth: May 10, 1978

Place of birth: Vienna, Austria

Nationality: Austrian

Professional Experience

2000-2004	Study of Microbiology and Genetics with the main topic in Molecular Microbiology at the University of Vienna
2004-2006	Diploma thesis ('Molecular Analysis of Antigenic Variation in *Mycoplasma agalactiae*') at the Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine Vienna, *Mycoplasma* research group
2006	Master of Science (with honours)
2006-2010	Dissertation at the Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine Vienna, *Mycoplasma* research group

Publications

Research paper

Stefan Czurda, Wolfgang Jechlinger, Renate Rosengarten, and Rohini Chopra-Dewasthaly. Xer1-Mediated Site-Specific DNA Inversions and Excisions in *Mycoplasma agalactiae* J. Bacteriol. published ahead of print on 18 June 2010, doi:10.1128/JB.01537-09

Posters

Stefan Czurda*, Rohini Chopra-Dewasthaly, Christine Citti, Renate Rosengarten, Wolfgang Jechlinger (2006). Function of the Xer1 recombinase of *Mycoplasma agalactiae* in *Escherichia coli*.

16th International Congress of the International Organization for Mycoplasmology (IOM), Cambridge, UK, July 9th-14th 2006

Stefan Czurda*, Rohini Chopra-Dewasthaly, Christine Citti, Renate Rosengarten, Wolfgang Jechlinger (2008). The rate of Vpma switching in *Mycoplasma agalactiae* is influenced by sequences flanking the specific Xer1 recombination-site. 17th International Congress of the International Organization for Mycoplasmology (IOM), Tianjin, P.R. China, July 6th-11th 2008

Rohini Chopra-Dewasthaly*, **Stefan Czurda**, Martina Zimmermann, Joachim Spergser, Renate Rosengarten (2010). The many facets of the Vpma antigenic variation system of *Mycoplasma agalactiae*. 110th General meeting of ASM, San Diego, CA, USA, May 23th-27th 2010

Stefan Czurda, Shivanand Hegde, Martina Zimmermann, Renate Rosengarten, and Rohini Chopra-Dewasthaly* (2010). Xer1-independent mechanisms of Vpma phase variation are triggered by the host immune response. 17th International Congress of the International Organization for Mycoplasmology, Chianciano Terme, Siena, Italy, July 11th-16th 2010

Oral presentations

Stefan Czurda*, Rohini Chopra-Dewasthaly, Christine Citti, Renate Rosengarten, Wolfgang Jechlinger (2007). Die Xer1 Rekombinase von *Mycoplasma agalactiae*: Inversionen und Exzisionen. 6. deutschsprachiges Mykoplasmen-Symposium, Fürth, Deutschland, 28.Juni - 1.Juli 2007

Stefan Czurda*, Wolfgang Jechlinger, Christine Citti, Renate Rosengarten, Rohini Chopra-Dewasthaly (2009). Identifikation von ortsspezifischen DNA-Exzisionen in *Mycoplasma agalactiae* mithilfe des *lacZ*-Reportergens. 7. deutschsprachiges Mykoplasmen-Symposium, Morschach, Schweiz, 25. - 27.Juni 2009

Stefan Czurda, Wolfgang Jechlinger, Christine Citti, Renate Rosengarten, and Rohini Chopra-Dewasthaly* (2009). Identification of Xer1-mediated excisions in *Mycoplasma agalactiae* by a *lacZ*-based reporter system. Mycoplasmology - A Review of developments over the last decade, Gran Canaria, Spain, June 10^{th}-12^{th} 2009

Stefan Czurda*, Wolfgang Jechlinger, Renate Rosengarten, Rohini Chopra-Dewasthaly (2010). Xer1-mediated site-specific DNA inversions and excisions in *Mycoplasma agalactiae*. 32. Jahrestagung der Österreichischen Gesellschaft für Hygiene, Mikrobiologie und Präventivmedizin (ÖGHMP), Wien, Österreich, 17. - 20.Mai 2010

Rohini Chopra-Dewasthaly*, **Stefan Czurda**, Shivanand Hegde, Martina Zimmermann, Renate Rosengarten (2010). The many facets of Vpma surface antigenic variation in *Mycoplasma agalactiae*. 32. Jahrestagung der Österreichischen Gesellschaft für Hygiene, Mikrobiologie und Präventivmedizin (ÖGHMP), Wien, Österreich, 17. - 20.Mai 2010

*presenting author