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Original article

Validation of the suppressive subtractive hybridization method in *Mycoplasma agalactiae* species by the comparison of a field strain with the type strain PG2

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Abstract – The subtractive suppressive hybridization (SSH), a method that allows the identification of sequences that are present in one genome (tester) but not in the other (driver), is a promising technique for the comparison of *Mycoplasma agalactiae* pathogenic strains. The optimal conditions for SSH were established by subtracting the *M. agalactiae* type strain PG2 DNA from the *M. agalactiae* strain 5632 DNA. Because these two strains possess different *vpma* gene repertoires, 5632-specific *vpma* sequences (and possibly other 5632-specific sequences) were predicted to be retrieved by SSH. The subtracted tester DNA was PCR-amplified and cloned into the pGEM-T easy *E. coli* vector. Two independent libraries were generated and used to prepare individual probes that were tested by Southern blot with genomic DNA from various field isolates and mycoplasma reference strains. Sequence analysis of two overlapping clones showed that they potentially code for a large carboxyterminal portion of a new *vpma* ORF. Several DNA fragments homologous to insertion sequences were also found in 5632 and related strains. These preliminary data suggest that SSH is a powerful method to investigate differences between mycoplasma strains, and may be applied to molecular epidemiology, diagnostic, and host specificity or pathogenicity determinant discovery.

***Mycoplasma agalactiae* / subtractive hybridization / *vpma* / insertion sequence**

1. INTRODUCTION

Mycoplasma agalactiae is one of the causal agents of the "contagious agalactia" syndrome in ovine and caprine species, usually characterized by mastitis in lactating animals, arthritis and conjunctivitis. In most cases, the host spontaneously recovers from the disease symptoms but remains chronically infected with long term shedding of *M. agalactiae* in the milk or other body secretions [5].

Antigenic and genomic analysis of diverse *M. agalactiae* strains or field isolates

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has shown a strong heterogeneity between virulent isolates [4, 11, 14, 19]; experimental and field studies suggest that certain *M. agalactiae* strains have a lower virulence compared to the fully pathogenic strains [23]. Comparison of selected *M. agalactiae* strains should help to understand the molecular basis of these differences.

Suppressive subtractive hybridization (SSH) is an efficient method to find DNA fragments that are present in one organism (tester) and not in the other (driver). SSH has been applied to various biodiversity surveys, molecular epidemiology studies, and identification of species-specific markers or virulence factors in diverse bacterial species [1, 2, 6, 17, 21, 29].

One of the goals of our laboratory is to apply this method to mycoplasmas, in order to compare strains having different phenotypic characteristics, such as pathogenicity or surface antigen compositions, or in order to compare phylogenically related mycoplasma species. This approach could lead to the discovery of new molecular epidemiology markers, virulence factors, or host specificity determinants. This could also help to understand mycoplasma genome plasticity within or between species. This method might also be applied to study mycoplasma gene expression in different environments, especially during the course of infection.

Since SSH has never been used in mycoplasmas, we performed a pilot study to implement this method in our laboratory. Here we present the validation of SSH for *M. agalactiae* and preliminary results obtained when comparing the type strain PG2 and the field isolate 5632 of this species. These two strains were chosen because various studies have suggested the existence of differences in their genomes. The 5632 strain has also been shown to differ from PG2 by the lack of surface antigen(s) recognized by the 1D4 monoclonal antibody in western or colony immunoblots [4], the presence of an insertion sequence, IS*Mag1* [19], the Dam-like methylation of its DNA and the composition of *vpma* gene repertoires [14]. The *M. agalactiae* type strain PG2 harbors a family of genes coding for abundant surface lipoproteins (Vpmas) that undergo high frequency phase variation; the *vpma* locus of the clone 55-5 of PG2 contains 6 *vpma* genes [13, 15]. The degenerate oligonucleotide probe A3F can specifically hybridize with a conserved sequence that is present in the *vpma* genes. Various *M. agalactiae* strains or field isolates appear to harbor various *vpma* gene repertoires in Southern experiments using A3F and 6 *vpma*-specific probes [14]. Interestingly, the 5632 strain is suspected to harbor at least two uncharacterized members of the *vpma* gene family that are not present in the type strain PG2, as shown by the presence of two A3F-reactive bands that were not recognized by any of the PG2-*vpma* specific probes in 5632 genomic DNA. The 5632 and PG2 strains were therefore chosen in our first attempt to apply SSH to phenotypically different strains of *M. agalactiae*.

2. MATERIALS AND METHODS

2.1. Strains

The mycoplasma type strains and field isolates used in this study are part of a collection maintained by F. Poumarat in AFSSA, Lyon (France) and are listed in Table I. Species identification of *M. agalactiae* isolates was performed by the MF-dot immunobinding method [20] with specific rabbit antisera in AFSSA, Lyon (France). Mycoplasma cultures were propagated in SP-4 liquid medium [28] or Aluotto liquid medium [3] at 37 °C. To establish the strain collection, the mycoplasma cultures were serially diluted, plated on Aluotto-agar and a single colony was picked from each isolate, transferred into a fresh liquid medium, grown again and kept at –80 °C. Western blots with mycoplasma total protein preparations using the 1D4 monoclonal antibody were performed as described elsewhere [4] to determine their serotype.

Table I. Mycoplasma strains.

Name	Source	Animal	Clinic	Date	Origin	1D4[a]
M. agalactiae						
PG2	Type strain	caprine	typical	1973	Spain	+
9	milk	ovine			Italy	+
190					Rumania	+
209	milk	caprine	typical	1983	France	–
332	join	caprine?		1998	France	+
3990			typical		France	–
4021	tank milk	ovine	no symptoms	1988	France	+
4025	tank milk	ovine	no symptoms	1988	France	+
4054	milk	caprine	typical	1986	France	–
4055	milk	caprine	typical	1987	France	–
4210	milk	caprine	typical	1982	France	+
4212			typical	1984	France	–
4258	milk	caprine	typical		France	+
5210			no symptoms	1988	France	+
5225	milk	caprine	typical	1991	Spain	+
5632	join	caprine	typical	1991	Spain	–
5670	milk	ovine	typical	1991	Spain	+
5725	milk	ovine	typical	1990	France	+
5826	milk	ovine	typical	1992	Spain	+
6833	milk	caprine	typical		Italy	+
6968	milk	ovine	typical	1993	Spain	+
7169	milk	caprine	typical		Switzerland	+
7314	milk	ovine	typical	1986	Greece	+
7327	milk	caprine	typical	1987	Greece	+
7375	milk	caprine	typical		Switzerland	+
7784	milk	ovine	typical	1994	France	+
8062	lung	caprine	atypical	1987	Ethiopia	–
8063			typical	1988	Turkey	–
8064	lung	ovine	atypical	1989	Ivory coast	–
8750	tank milk	ovine?			France	+
9385	milk	caprine	typical	< 1996	Portugal	+
9600				< 1996	Portugal	+
9875	milk	ovine		< 1996	Spain	+
M. bovis						
PG45	Type strain	bovine				ND

[a] Monoclonal antibody 1D4 reactivity was assessed by western blot.
ND: not done.

Table II. Oligonucleotides used.

Name	Sequence
RJ48	AGCACTCTCCAGCCTCTCACCGAGACCGACGTCGACTATCCATGAACG
Jbam12	GATCCGTTCATG
Jeco12	AATTCGTTCATG
RN48	AGCACTCTCCAGCCTCTCACCGAGAGGCAACTGTGCTATCCGAGGGAG
Nbam12	GATCCTCCCTCG
Neco12	AATTCTCCCTCG
R24	AGCACTCTCCAGCCTCTCACCGAG
J24	ACCGACGTCGACTATCCATGAACG
N24	AGGCAACTGTGCTATCCGAGGGAG
5632-vpma1-L1	AAACCAGCAGAAACACCAGG
5632-vpma1-R1	GTTCAGCCATCTTCTTTTGACA

The *Escherichia coli* DH5α strain (Invitrogen/Life-Technologies, Cergy-Pontoise, France) was grown in LB medium supplemented with the appropriate antibiotic.

2.2. Oligonucleotides

All oligonucleotides for adapter production and PCR were purchased from Invitrogen. The A1-bam and A1-eco adapters were constructed with the oligonucleotide RJ48 annealed to Jbam12 or Jeco12, respectively; the A2-bam and A2-eco adapters were constructed with the oligonucleotide RN48 annealed to Nbam12 or Neco12, respectively. Annealing conditions were set according to the manufacturer's instructions. Oligonucleotide sequences are given in Table II. The oligonucleotides R24, J24, N24, J12 and N12 were used for the comparison of *Neisseria meningitidis* and *Neisseria gonorrhoeae* by a subtractive hybridization technique, the Representational Difference Analysis (RDA) [26]; both the RDA and SSH methods are based on PCR enrichment of the target (tester-specific) sequences, but differ in their protocols. The oligonucleotides RJ48 and RN48 were designed by the concatenation of the R24 sequence with the J24 or the N24 sequence, respectively. The rationale of this was to give us the possibility to switch from SSH to RDA, if desired, just by using two extra 12-mer oligonucleotides, Reco12 and Rbam12, which have been described elsewhere [26], to obtain the set of adapters for RDA.

2.3. Molecular biology techniques

The PCR were performed on an MJresearch PT100 thermocycler, with the recombinant *Taq* polymerase and reaction buffer from Invitrogen, the plasmid preparations for sequencing were performed with the WizardSV miniprep system (Promega, Charbonnières, France). The sequencing reactions were performed by PCR amplification according to Applied Biosystems (Foster City, California, USA; Big Dye Terminator) or Amersham Pharmacia Biotech (Piscataway, New Jersey, USA; ET Terminator) protocols; the samples were processed onto capillary or slab gel sequencers by Genome-Express (Meylan, France). The ligation reactions were performed with the LigaFast ligation system (Promega). The pGEM-T-easy plasmid (Promega) was used according to the manufacturer's recommendations. For the dot-blot hybridization experiments, colony-PCR were performed directly on individual clones harboring the recombinant pGEM-T-easy vectors using the primers J24 and N24, with the following reaction conditions: final volume of 50 µL

with 5 µL of 10× reaction buffer, 200 µM dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 2.5 U *Taq* polymerase; 30 cycles of 30 s at 94 °C, 30 s at 49 °C and 1.5 min at 68 °C, plus a final extension of 7 min at 68 °C. The amplification products sizes were controlled by 1% agarose gel electrophoresis; 10 µL of each positive PCR reaction was mixed with 90 µL of 0.1 M NaOH, and 5 µL of the mix were spotted onto a series of nylon membranes (Roche, Meylan, France). After rinsing in 2× SSC, the membranes were UV-cross linked and processed as described for the Southern blots (see below).

For the 5632-*vpmaA* specific PCR, the following conditions were used: final volume of 50 µL with 5 µL of 10× reaction buffer, 200 µM dNTP, 1.5 mM $MgCl_2$, 0.2 µM of primers 5632-vpma1-L1 and R1, 2.5 U *Taq* polymerase; 30 cycles of 30 s at 94 °C, 30 s at 46 °C and 30 s at 68 °C, plus a final extension of 7 min at 68 °C.

2.4. DNA preparations

For the subtraction experiments, the tester and driver mycoplasma genomic DNA were prepared by proteinase K digestion and phenol-chloroform extraction. For other applications, the genomic DNA were prepared by a rapid method [7].

2.5. Suppressive Subtractive Hybridization

The Suppressive Subtractive Hybridization technique [10] adapted to the prokaryotic genome study [2, 14] was used on mycoplasma strains as follows: PG2 driver DNA (2 µg) and 5632 tester DNA (2 µg) were digested to completion by *Sau*3AI or partially by *Tsp*509I (NEB) in a 50 µL final volume. After a 25/24/1 phenol/chloroform/isoamyl alcohol extraction, DNA were ethanol-precipitated, resuspended in 10 µL water, and their concentration was estimated on a 1% agarose gel. Two subsets of the digested tester DNA (100 ng) were separately ligated overnight at 16 °C with the adapters A1 or A2 (20 pmol) in a 10 µL final volume by T4 DNA ligase. For *Sau*3AI-digested tester DNA, adapters A1-bam and A2-bam were used, whereas for *Tsp*509I-digested tester DNA, adapters A1-eco and A2-eco were used. The ligase was heat-inactivated 5 min at 72 °C. A portion of each adapter-ligated tester DNA subset (10 ng) was mixed with an excess of digested driver DNA (600 ng) in hybridization buffer (50 mM HEPES-HCl pH 8.0; 0.5 M NaCl; 0.2 mM EDTA pH 8.0, final concentrations), in a final volume of 4 µL, denatured for 1.5 min at 98 °C, and then incubated for 1.5 h at 55 °C. The two subsets were then simultaneously mixed together with 300 ng of freshly denatured driver DNA in hybridization buffer, and incubated for 18 h at 55 °C in a final volume of 10 µL. The subtracted mix was diluted with 200 µL of dilution buffer (20 mM HEPES-HCl pH 8.3; 50 mM NaCl; 0.2 mM EDTA pH 8.0). A nested PCR was then performed. For the first PCR, the R24 primer was used; reaction conditions were as follows: final volume 50 µL with 5 µL of 10× reaction buffer, 1 µL of the diluted subtracted DNA as the template, 200 µM dNTP, 1.5 mM $MgCl_2$, 0.4 µM primer, 2.5 U *Taq* polymerase; a manual hot start of 2 min at 72 °C was first performed, followed by 26 cycles of 30 s at 94 °C, 30 s at 52 °C and 2 min at 68 °C, plus a final extension of 10 min at 68 °C. For the second PCR, the primers J24 and N24 were used; reaction conditions were as follows: final volume 50 µL with 5 µL of 10× reaction buffer, 1 µL of the first PCR mix diluted 1/40 in water as template; 200 µM dNTP, 1.5 mM $MgCl_2$, 0.2 µM primers, 2.5 U *Taq* polymerase; 23 cycles of 30 s at 94 °C, 30 s at 49 °C and 2 min at 68 °C, plus a final extension of 10 min at 68 °C.

2.6. Construction of libraries

After the nested PCR, approximately 10 ng of PCR final products from the 5632-PG2 SSH were directly ligated with

12 ng of pGEM-T-easy in a 10 µL final volume. Half of the ligation mix was used to transform RbCl-competent DH5α *E. coli*.

2.7. Southern experiments

For Southern experiments, 1 µg of genomic DNA was digested with *Eco*RI, *Hin*dIII or *Ase*I, submitted to 1% agarose gel electrophoresis and transferred onto nylon membranes (Roche). The digoxigenin (Dig) labeling and detection systems (Roche) were used according to the manufacturer. The probes were labeled by Dig-11dUTP-PCR using the primers J24 and N24 and incubated with the membranes overnight in Church Buffer [8] at 60 °C; the membranes were briefly rinsed with 0.2× SSC–0.1% SDS at room temperature and washed in the same solution for 1 h at 60 °C. Detection of hybridized probes was performed with the alkaline phosphatase-conjugated anti-Dig Fab antibodies and CDPstar reagents (Roche). The A3F oligonucleotide probe and the PG2-*vpmaU-Z* specific probes were used as described elsewhere [13–15].

2.8. Computer analysis of the sequences

The BLAST suite of the programs (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) was used for sequence homology searches on non redundant databases. Multiple alignments were performed with the Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html) [9, 14] or Clustal W 1.8 (http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl) programs [25] at the Infobiogen website. Sequences were analyzed for the presence of ORF by the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) at the NCBI website.

2.9. Sequence accession numbers

The A36, C5, C11, C12, C28 and C30 insert sequences are available at the GenBank database under accession numbers BZ630067, BZ689859, BZ630068, CC875160, BZ630069 and BZ630070, respectively.

3. RESULTS

3.1. Construction of two *M. agalactiae* 5632-specific libraries

The SSH was performed using 5632 DNA as a tester and PG2 DNA as a driver in two independent experiments, one using *Tsp*509I digested DNA, the second using *Sau*3AI digested DNA. The following controls were performed along with the *Sau*3AI-based subtraction. Control No. 1: in a parallel SSH experiment, the 5632 tester DNA was subtracted by itself (i.e. by the 5632 DNA used as a driver DNA); control No. 2: the driver (PG2) and tester (5632) DNA were mixed and processed as in the normal SSH experiment, except that the denaturing and hybridization steps were omitted and replaced by 2 h of incubation at room temperature before the nested PCR; control No. 3: the 5632 tester DNA was processed in the same conditions as in control No. 2, except that no driver DNA was added to the tubes; control No. 4 the driver DNA from PG2 or 5632 were processed in the same conditions as in control No. 2, except that no tester DNA was added to the tubes. As expected, only the 5632-PG2 SSH gave visible PCR products, ranging from 200 to 700 bp, mostly representing tester-specific fragments and not background or unspecific amplification.

The final PCR products were cloned in *E. coli*, giving approximately 10^3 colonies per transformation, and independent colonies were randomly picked to give two libraries of 48 clones each, named "A" and "C", corresponding respectively to the *Tsp*509I and *Sau*3AI-driven SSH experiments.

The PCR insert carried by each clone was PCR-amplified directly from the *E. coli* cultures, using primers J24 and N24, and

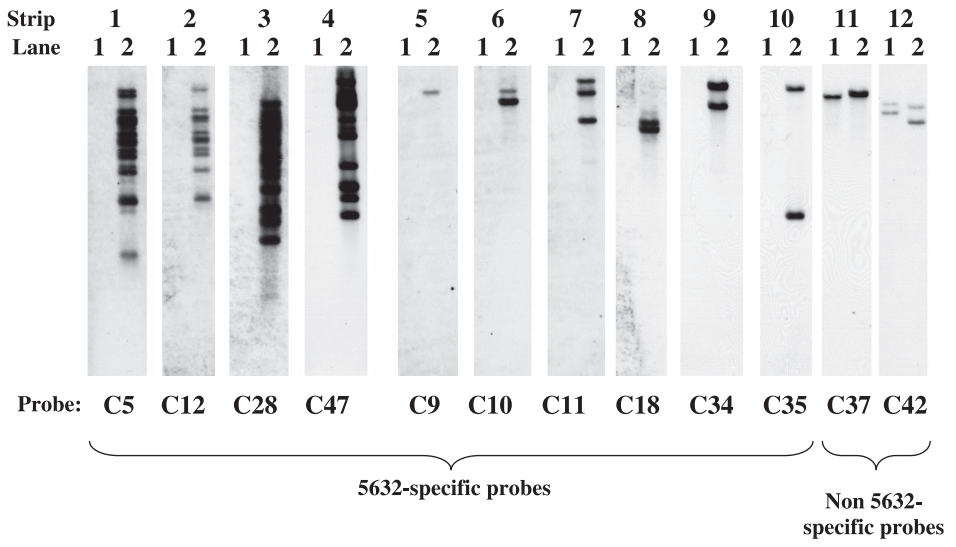

Figure 1. Screening of *M. agalactiae* subtractive libraries for tester-specific fragments. Following the subtraction by the PG2 driver DNA, the 5632 tester DNA was PCR-amplified and the products were cloned *en masse* into *E. coli*; individual probes were prepared from cloned PCR fragments and incubated with a series of nylon membrane strips obtained by Southern transfer of *Eco*RI-digested genomic DNA from PG2 (lanes 1) and 5632 (lanes 2) strains. Twelve strips are depicted as examples of specific (strips 1-10) or unspecific (strips 11 and 12) hybridizations with various probes, as indicated below each strip.

the sizes of the inserts were estimated to range between 200 and 700 bp on agarose gel. In the libraries A and C, respectively 30 and 47 clones gave a positive amplification, with a single band larger than 300 bp; the rest of the clones gave either no amplification or multiple amplification products, and were discarded from the study. Each selected clone was used as a template to generate 77 individual probes by PCR-Dig labeling, which were tested on Southern nylon membrane strips carrying genomic DNA digests from the tester and the driver (Fig. 1). The libraries A and C contained respectively 14 and 32 tester-specific clones.

3.2. Identification of *vpma*-like genes in *M. agalactiae* 5632

The strain 5632 is suspected to contain at least two *vpma*-related genes that are not present in the type strain PG2, as shown by the presence of two A3F-reactive bands that were not recognized by any of the PG2-*vpma* specific probes in 5632 genomic DNA (Fig. 2A). Sequence analysis of 5632-specific fragments from the "A" library by BlastX homology searches revealed that the insert carried by the A36 clone partially matched with the aminoterminal portion of the *vpmaY* gene product [13] and other variable surface proteins from *M. agalactiae* PG2 and *Mycoplasma bovis*. The nucleotides 2–100 of the 310-bp A36 insert were potentially coding for a 33-a.a. peptide having 81% identity with the a.a. 18–50 of VpmaY and possessing the consensus acylation/peptidase II cleavage motif AAKC found in the Vpma family. The nucleotide positions 20–39 of A36 were found to correspond to the degenerate oligonucleotide sequence A3F, with 2 mismatches at the positions 27 and 30 (Fig. 2B). Note that no *Ase*I restriction site is present in the A36 sequence, which implies that the A36 probe

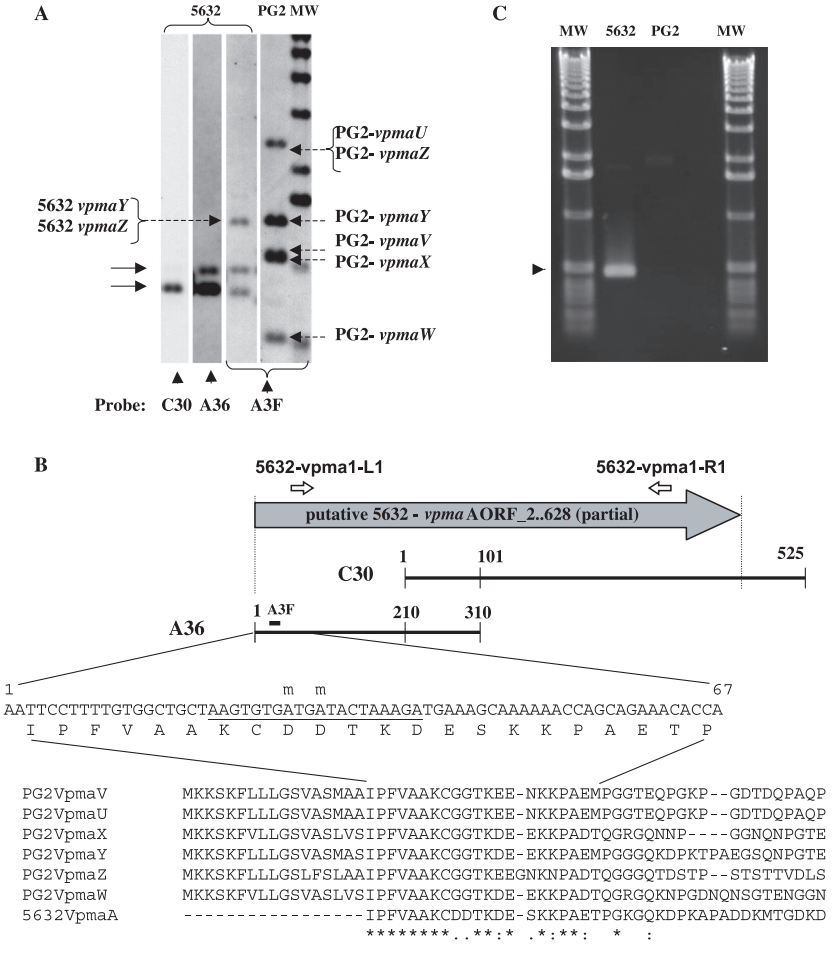

Figure 2. A putative new member of the *vpma* family. (A) Southern-blot analysis of *Ase*I-digested PG2 and 5632 genomic DNA (as indicated above the blots) with A3F, C30 and A36 probes (as indicated below the blots). The dotted arrows indicate the A3F-reactive fragments that are also recognized by the PG2-*vpma* specific probes in the PG2 or 5632 genomic DNA. The solid arrows indicate the A3F-reactive fragments of 5632 that are not recognized by any of the 6 PG2-*vpma* specific probes. The probes C30 and A36 both hybridize with the lower fragment (lower solid arrow). MW: 12-kb marker. (B) Sequence analysis of the assembled A36 and C30 clones: the grey arrow represents the putative partial ORF, as deduced from the nucleotides 2 to 628. The region homologous to the conserved N-terminal part of the *M. agalactiae* PG2-Vpmas is indicated on the sequence below. The A36 DNA insert harbors a nucleotide sequence recognized by the degenerate oligonucleotide probe A3F (underlined), with only two mismatches (m). On the multiple alignment with Vpmas, "*" indicates positions which have a single, fully conserved residue, ":" indicates that a "strong" group of residues is fully conserved and "." indicates that a "weaker" group is fully conserved, according to the amino-acid group definitions in the ClustalW 1.8 manual file. (C) Colinearity of the putative 5632-*vpma* ORF to the genome of 5632: a 482-bp DNA fragment (arrowhead) was PCR-amplified using 5632, but not PG2, genomic DNA as the template (as indicated above the gel); the position of the PCR primers 5632-vpma1-L1 and R1 relative to the assembled A36 and C30 sequences is indicated by the open arrows on the same figure, panel B. MW: 12-kb marker.

cross-hybridizes with two distinct A3F-reactive fragments in *Ase*I-digested genomic DNA.

The A36 probe was then used to screen the "C" library by dot-blot hybridization, in order to find overlapping clones. One 5632-specific clone, C30, was recognized by the A36 probe and was further analyzed. In Southern blot with *Ase*I-digested 5632 genomic DNA, the C30 and A36 probes hybridized with an A3F-reactive band that was not recognized by any of the PG2-*vpma* specific probes (Fig. 2A).

Sequence analysis revealed that the nucleotides 1–101 of insert C30 perfectly overlap the nucleotides 210–310 of insert A36; the assembled sequence of A36 and C30 inserts was analyzed by the ORF finder program and the nucleotide positions 2 to 625 were shown to potentially encode a 205-a.a. carboxyterminal end of a putative ORF product (Fig. 2B), that was named 5632-VpmaA. The comparison of the putative 5632-VpmaA partial sequence with protein sequence databanks by the BlastP program confirmed the homologies between the a.a. position 1–33 of the 5632-VpmaA and the aminoterminal part of the PG2-Vpmas; other significant homologies were not found in the rest of the 5632-VpmaA sequence.

Two primers, 5632-vpma1-R1 and 5632-vpma1-L1, were designed to produce a 482-bp PCR amplicon from the 5632 genomic DNA that encompasses the overlapping portion of A36 and C30 inserts (Fig. 2C), indicating that the assembled sequences from the A36 and C30 clones are co-linear to the corresponding genomic region of *M. agalactiae*. No amplification was observed with the PG2 genomic DNA, further confirming that the putative 5632-*vpmaA* ORF is specific to the 5632 strain.

3.3. Identification of insertion sequences (IS)-related sequences in *M. agalactiae* 5632

During the screening of the A and C libraries, out of a total of 46 tester-specific fragments, four probes recognized multiple band patterns (from 10 to more than 20 bands) in *Eco*RI-digested 5632 genomic DNA (see Fig. 1, probes C5, C12, C28, C47). Amongst the 77 probes from the libraries A and C that were screened for tester-specific sequences, a further five probes were found to recognize the various multiple band patterns in 5632 *Eco*RI-digested DNA, comprising from 5 to 15 bands, but these probes also recognized one or several bands in the PG2 *Eco*RI-digested DNA and were therefore not selected for ongoing studies. The insert sequences from the clones C5, C12 and C28 were found to be almost identical to three contiguous *Sau*3AI segments of the IS*Mag1* gene from *M. agalactiae* strain 3990 (GenBank AJ311887), that is also present in 5632 but not in PG2 [19]. The C47 clone sequence was found to be unrelated to IS*Mag1*. Southern analysis of a collection of *Hind*III-digested DNA from 33 *M. agalactiae* strains including PG2 revealed that the C28 probe recognized a high number of restriction fragments in 5632 and in four other isolates, as well as a single band in a further two isolates (Fig. 3). Within our collection, all the strains that have been previously described to harbor the IS*Mag1* were also found to be C28-reactive, with hybridization patterns identical to the previously published ones.

During the sequencing of several other 5632-specific fragments, the C11 clone was found to be almost identical to a segment of the IS*30*-like element present in *M. bovis* (GenBank AF396969), which is associated to the *vsp* locus of PG45. The C11 probe reacted with three distinct bands in *Eco*RI-digested 5632 DNA, a pattern which clearly differed from the 15–20 bands recognized by the probes C5, C12, C28 and C47. Southern analysis of the *M. agalactiae* strain collection digested by *Hin*dIII revealed that the C11 probe recognized several bands in 5632 and four other strains, as well as a single band in two strains (Fig. 3). Within the *M. agalactiae* collection, the C28 and C11 positive strains fell into two overlapping but not identical

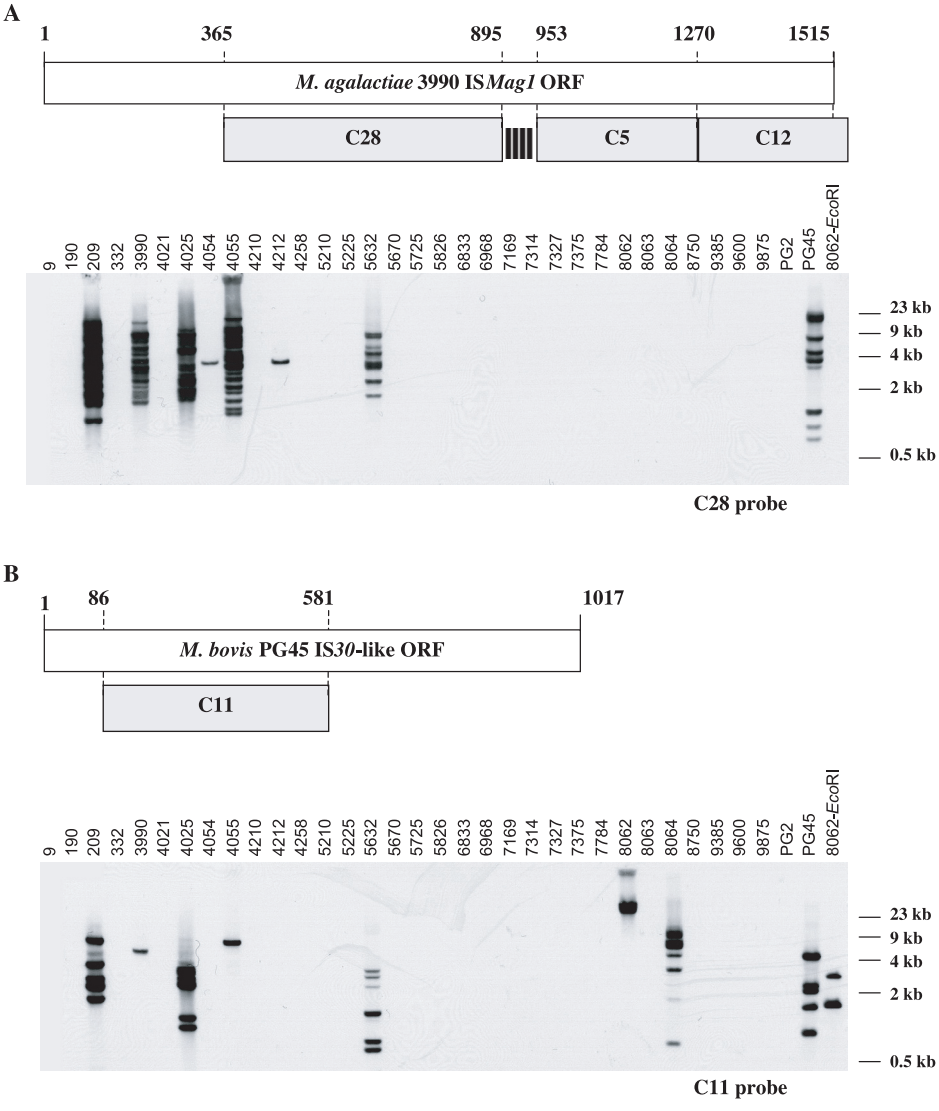

Figure 3. Genomic DNA fragments from *M. agalactiae* 5632 that are homologous to insertion sequences, and related Southern analysis of *Hin*dIII-digested genomic DNA from 32 *M. agalactiae* field isolates, and from *M. agalactiae* PG2 and *M. bovis* PG45 type strains. Note that 8062 genomic DNA (lane No. 26 from the left) is not sensitive to *Hin*dIII digestion and was therefore also digested by *Eco*RI (last lane). (A) The available sequences of clones C5 and C28 (grey boxes) from 5632 correspond to the nucleotide positions 365–895 and 953–1270, respectively, of two *Sau*3AI adjacent fragments in the IS*Mag1* sequence from *M. agalactiae* strain 3990 (GenBank AJ311887). Note that the C28 clone was only partially sequenced over 531 bp; the hatched box represents unavailable sequence data. (B) The sequence of clone C11 (grey box) from 5632 corresponds to the nucleotide positions 86–581 of a *Sau*3AI-fragment in the IS*30*-like DNA sequence from the *M. bovis* PG45 strain (GenBank AF396969).

groups: five strains (209, 3990, 4025, 4055 and 5632) appeared to carry both C28 and C11 sequences, whereas the strains 4054 and 4212 carried only the C28 sequence and strains 8062 and 8064 carried only the C11 sequences. The *M. bovis* type strain PG45 harbored both C11 and C28 sequences, as expected.

4. DISCUSSION

The application of the SSH to a prokaryotic genome study was originally performed with the gram negative gastric pathogen *Helicobacter pylori* [2]; the method was therefore slightly modified in order to adapt it to the mycoplasmas characteristics. Because of the low GC% of mycoplasmas, with *M. agalactiae* strain PG2 having an average of 33 GC% [12], the hybridization temperature (originally 63 °C) was lowered to 55 °C. After the subtraction step, a nested PCR was performed and amplified products were visualized on 1% agarose gel as a smear ranging from 0.3 to 1 kb. It was necessary to increase the number of cycles (originally 16) up to 23 in the second PCR to obtain visible amplification products. Because of this higher number of cycles, several negative controls were performed, in order to check that unwanted amplification did not occur during the nested PCR. Subtractive hybridization proved to be a useful method to investigate the differences between mycoplasma field isolates. Sequences that were predicted to be recovered by SSH were effectively found by screening a minimal number of clones from only two libraries, showing the efficiency of this method; furthermore, highly repeated sequences were not over-represented in the libraries, allowing an easier screening and characterization of the tester-specific sequences.

Two overlapping *vpma* related DNA fragments were found in two independent 5632-specific libraries, which allowed the identification of a potential new *vpma* ORF carboxyterminal part. Additional clones carrying the potential aminoterminal end of this putative 5632-*vpmaA* ORF were not found, probably because they correspond to highly conserved portions of *vpma* genes and were therefore subtracted by their counterparts in PG2. Whether other 5632-*vpma* genes are present in the 5632 strain genome and if the putative corresponding 5632-Vpmas undergo phase variation in a way similar to the PG2-Vpmas remains to be investigated. Because the A36 sequence is devoid of the *Ase*I restriction site, the fact that the A36 probe recognized two A3F-reactive *Ase*I fragments in the Southern blot suggests that at least one other member of the *vpma* family should be present in the 5632 genome.

The mycoplasma genomes are shown to contain diverse repeated sequences (like IS elements, or multiple tandem repeats present in certain genes like the *vpmas* or *vsps*), that are prone to be over-represented in a classic subtractive hybridization library. The IS*Mag1* appears to have a very high number of copies in certain *M. agalactiae* strains like 3990 or 5632, as shown by Southern experiments, with a minimum of 30 copies per genome [19]. Assuming that the chromosome size of *M. agalactiae* strain 5632 is roughly equivalent to the one of PG2, i.e. 945 kb [27], approximately 5% of the 5632 genomic DNA should correspond to IS*Mag1* sequences. An attempt to compare the genomes of PG2 and 15 unspecified *M. agalactiae* strains by DNA-DNA hybridizations [16] suggests that, in certain genetic loci, differences ranging from 40% to 65% relative to PG2 might exist, but the exact genomic difference between 5632 and PG2 has never been investigated. It is commonly admitted that, even in low stringency conditions, two prokaryotic single stranded DNA will form heteroduplexes only if they show more than 80% sequence complementarity, and that two bacterial strains belonging to a given species share more than 70% of relative binding ratio of DNA (which means that a maximum of 30% of their DNA might not form heteroduplexes,

due to sequence identities ranging from 0% to 80% between the DNA) [22]. It should also be noticed that the PG2 and 5632 strains have been classified within the same species according to various phenotypic and genotypic traits [18]. Therefore, depending on the degree of relatedness between 5632 and PG2 genomes, it is reasonable to expect that the proportion of IS*Mag1*-related sequences in a 5632-specific library generated by means other than SSH would be higher than 15%. The low amounts of such clones obtained during this study (amongst the 46 sequences that were 5632-specific, only 3, namely C5, C12 and C28, were related to the IS*Mag1*) might be attributed to the SSH technique [10], which equalizes the concentrations between abundant and non abundant tester-specific DNA fragments, keeping the repeated sequences like IS*Mag1* from being over-represented in the resulting libraries.

The presence of IS*Mag1* was tested by Southern hybridization on 26 *M. agalactiae* strains, with a 1.5-kb probe encompassing the entire IS sequence [19]; it was suggested that the presence of IS*Mag1* correlates with the strain serotype as assessed by its reactivity with the monoclonal antibody 1D4, i.e. only 1D4-negative strains harbor the IS*Mag1*. Here, we screened a collection of 32 *M. agalactiae* field isolates, including the 26 ones previously described, with the 0.5-kb C28 probe corresponding to a *Sau*3AI internal fragment, and we confirmed these results. The strain 4025 appears to have a special status, since its total protein preparation was recognized by the 1D4 monoclonal antibody (Tab. I), whereas its genomic DNA was C28-positive. These findings indicate that the correlation between the presence of IS*Mag1* and the absence of the 1D4 epitope is not absolute and raise the question of possible DNA transfer systems between strains, within the *M. agalactiae* species, or between different species. The occurrence of the gene transfer phenomena between mycoplasmas has been shown in *M. pulmonis* [24]; this should be considered when using molecular markers for phylogeny and epidemiology studies. Furthermore, the presence of at least two IS-related sequences that are both found in the *M. bovis* type strain PG45 and in a specific subset of *M. agalactiae* strains clearly illustrates the importance of setting up accurate diagnostic tools for mycoplasma species identification.

We demonstrated that the SSH method can be easily and efficiently applied to a mycoplasma genome comparison. The validation of this technique now opens a wide variety of promising applications, ranging from basic species-specific diagnostic assays or molecular epidemiology, to more complex investigation of gene expression or discovery of new virulence factors.

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