

Development of serological proteome analysis of mastitis by *Staphylococcus aureus* in ewes.

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Abstract: *Staphylococcus aureus* is a major agent of mastitis in ruminants worldwide. So far, efficient measures for its prophylaxis (including vaccination) have proven unsuccessful and there is a need for a better understanding of the host response to udder infection by *S. aureus*. Serological proteome analysis (SERPA) is a promising technique that can be used to identify *S. aureus* immunodominant determinants providing that bacterial culture conditions used to grow *S. aureus* strains for protein sample preparation mimic the context of mastitis. A *S. aureus* strain was used in experimental mastitis to generate sheep serum used to determine the best growth conditions for SERPA. Sera collected in the field from different ewes suffering from mastitis by *S. aureus* were used to confirm experimental observations. Three different culture media (BHI, whey and iron-depleted RPMI) were tested. The influence of aeration and growth phase on protein production was also evaluated by immunodetection of protein samples prepared from cultures grown in different conditions and obtained from different culture fractions (supernatant, cell wall, and total lysates). Our results showed that culturing in iron-depleted RPMI with (secreted proteins, prepared from stationary phase) or without aeration (cell wall proteins, prepared from early stationary phase, and total proteins, prepared from exponential phase) is the condition that best mimics growth in vivo during mastitis and this in vitro growth condition is to be used henceforth in experiments involving SERPA.

1 **Development of serological proteome analysis of mastitis by *Staphylococcus***
2 ***aureus* in ewes.**

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1 **Abstract.**

2 *Staphylococcus aureus* is a major agent of mastitis in ruminants worldwide. So far, efficient
3 measures for its prophylaxis (including vaccination) have proven unsuccessful and there is a
4 need for a better understanding of the host response to udder infection by *S. aureus*.
5 Serological proteome analysis (SERPA) is a promising technique that can be used to identify
6 *S. aureus* immunodominant determinants providing that bacterial culture conditions used to
7 grow *S. aureus* strains for protein sample preparation mimic the context of mastitis. A *S.*
8 *aureus* strain was used in experimental mastitis to generate sheep serum used to determine the
9 best growth conditions for SERPA. Sera collected in the field from different ewes suffering
10 from mastitis by *S. aureus* were used to confirm experimental observations. Three different
11 culture media (BHI, whey and iron-depleted RPMI) were tested. The influence of aeration and
12 growth phase on protein production was also evaluated by immuno-detection of protein
13 samples prepared from cultures grown in different conditions and obtained from different
14 culture fractions (supernatant, cell wall, and total lysates). Our results showed that culturing in
15 iron-depleted RPMI with (secreted proteins, prepared from stationary phase) or without
16 aeration (cell wall proteins, prepared from early stationary phase, and total proteins, prepared
17 from exponential phase) is the condition that best mimics growth *in vivo* during mastitis and
18 this *in vitro* growth condition is to be used henceforth in experiments involving SERPA.

1 Introduction

2 *Staphylococcus aureus* is one of the major causative agents of mastitis in ruminants
3 (Bergonier and Berthelot, 2003; Sutra et al., 1990). The impact of *S. aureus* mastitis on milk
4 production is very significant however, the problem is currently hard to tackle for mastitis in
5 dairy cows, sheep and goats. It is typically refractory to antibiotic treatment and prophylactic
6 measures, including the development of an effective vaccine, have so far proven unsuccessful
7 for the control of this disease. Serological proteome analysis (SERPA) is a promising
8 technique that can be used to shed light on the host's immune response to a staphylococcal
9 infection and that was used to mine new antigen candidates for vaccine development in
10 human infections (Vytvytska et al., 2002). SERPA is a combination of 2 dimensional gel
11 electrophoresis (2-DE), immunoblotting and spot sequencing. Proteins extracted from *S.*
12 *aureus* cultures are separated in 2-DE, transferred onto membranes and exposed to sera from
13 either contaminated or healthy animals. Staphylococcal proteins that are immunogenic are
14 recognized by the serum and can then be determined and characterized. However, protein
15 sample preparation cannot be carried out directly from a sample obtained from a site of
16 infection because the number of bacterial cells present in an infection is too small compared
17 to that required for the preparation of a sample to be submitted to 2D gel electrophoresis.
18 Protein samples therefore have to be prepared from *in vitro* cultures which leads to the
19 problem that proteins potentially recognized by the serum (which is directed against proteins
20 produced *in vivo*, during an infection) may not be present when the bacterial strain is cultured
21 *in vitro*. Indeed, antigen production is different when *S. aureus* is grown *in vitro* and *in vivo*
22 (Watson and Prideaux, 1979). It is thus important to find bacterial growth conditions that best
23 mimic mastitis. In this study, experimental mastites were carried out in ewes using a well-
24 characterized *S. aureus* O46 strain previously isolated from subclinical mastitis in a ewe. The
25 resulting sera were then used to analyze protein samples prepared from *S. aureus* cultures

1 grown on either whey, Roswell Park Memorial Institute medium (RPMI 1640) depleted of
2 iron or BHI with or without aeration, at different growth phases. Additional sera acquired in
3 the field were used to confirm the results obtained. Growth conditions were compared taking
4 into account the number of proteins detected and the strength of the signals obtained. We
5 hereby determine the *in vitro* growth conditions that enhance antigen synthesis and can be
6 used for studies of mastitis in ewes using SERPA.

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2 **Materials and Methods.**

3 **Bacterial strains.** *Staphylococcus aureus* strain O46 was previously isolated from a case of
4 ovine subclinical mastitis (Straub et al., 1999; Vautor et al., 2003). The genetic and genotypic
5 background of *S. aureus* O46 is well-documented and is representative of the major lineage
6 associated with mastitis in ewes in the southeast of France (Vautor et al., 2008).

7 *Staphylococcus xylosus*, *Lactobacillus helveticus*, *Streptococcus thermophilus*, and
8 *Escherichia coli* strains were used as negative controls for serum analysis. These bacterial
9 species were grown overnight in BHI broth (Scharlau, Barcelona, Spain) at 37°C with
10 agitation, MRS (Difco, Paris, France) at 30°C without agitation, M17 (Oxoid, Dardilly,
11 France) at 30°C without agitation and LB (Invitrogen, Cergy Pontoise, France) at 37°C with
12 agitation for *S. xylosus*, *L. helveticus*, *S. thermophilus* and *E. coli*, respectively.

13
14 **Preparation of whey.** The ewe milk used in this study was obtained from bulk milk,
15 collected at the very beginning of the lactation period of a dairy herd (ewe race Lacaune) in an
16 organic farm close to Rennes (Brittany, France). Ewe milk was microfiltered to remove
17 caseins so as to obtain “ideal whey” corresponding to the microfiltered permeate (MF).
18 Briefly, 50 L of ewe milk (Triballat, France) were skimmed, heated to 50°C and microfiltered
19 using a microfiltration pilot device equipped with a Pall Membralox 1P1940 membrane
20 having a 0.1 µm pore size cut-off. Ultrafiltered permeate (UF), where all protein compounds
21 are eliminated, was used to study secreted proteins and was prepared as described previously
22 (Ulve et al., 2008). The same batch of microfiltered and ultrafiltered milk was used
23 throughout this study.

1 **Growth conditions.** Strain O46 was cultured separately in three different media: BHI broth
2 (Scharlau), iron-depleted RPMI (Sigma, Saint Quentin fallavier, France) and microfiltered (or
3 ultrafiltered) milk. RPMI 1640 medium was depleted of iron (and hereafter referred to as iron-
4 depleted RPMI) by adding deferoxamine (0.15 mM) (Sigma). All cultures were performed as
5 follows: Overnight cultures in BHI were diluted 1:1000 in fresh medium. For aerobic
6 conditions, *S. aureus* O46 was grown in 500 mL flasks under agitation (150 rpm) at 37°C (a
7 flask-to-broth volumetric ratio of 5). For anaerobic conditions, *S. aureus* O46 was grown in
8 falcon tubes (50 ml) completely filled with medium and incubated at 37 °C without agitation.
9 Bacteria were either grown to early exponential phase, to early stationary phase or to late
10 stationary phase as determined by measurement of optical density at 600 nm (OD₆₀₀) and
11 plate counts on BHI agar using a previously described micromethod (Baron et al., 2006).

12
13 **Ewe infection.** Experimental mastitis was performed according to the French and European
14 laws on animal experimentations. *S. aureus* O46 strain was injected into both teats of a
15 primary lactating ewe 15 days after parturition. Briefly, urethral catheters (Portex® Jackson
16 Cat Catheter, Coveto, France) were inserted into the teat canal after a thorough disinfection of
17 the teat orifice with 70% ethanol. One millilitre of PBS containing 100 colony-forming-units
18 of *S. aureus* was injected through the catheter, and the catheter removed. Sera were prepared
19 from blood samples collected at D0, D7, D14 and D21 after injection. Briefly, blood samples
20 were stored during at least 2 hours before centrifugation (2600 rpm, 15 min). Sera were then
21 carefully taken and stored at -20°C.

22 Besides, sera obtained in the field from ewes suffering from *S. aureus* mastitis were collected
23 at various days after infection (not determined) and prepared similarly.

1 **Sample preparation for SDS-PAGE.** Total cell lysates and cell wall proteins were prepared
2 by treatment with lysostaphin as described by Vytvytska et al. with minor changes (Vytvytska
3 et al., 2002). After measurement of OD₆₀₀, 10⁹ bacteria were harvested and spun down (5000
4 x g, 5 min, 4 °C). The volume of culture medium was adjusted according to the type of
5 medium and growth phase to harvest 10⁹ bacteria. Cell pellet was washed twice with ice-cold
6 PBS and once with digestion buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 5 mM MgCl₂).
7 In order to obtain total cell lysates, cells (about 10⁹ cfu) were resuspended in 1 mL of
8 digestion buffer containing 1 mM PMSF, 10 µL of protease inhibitor cocktail (GE Healthcare,
9 Orsay, France) and 50 µg of lysostaphin (Sigma). After incubation at 37°C for 30 minutes,
10 cells were exposed to sonication (5 X 1min) and centrifuged at 10 000 x g during 10 minutes
11 at 4°C. The supernatant was stored at -20°C. In order to obtain surface proteins, after
12 washing, cells were resuspended in 1 mL digestion buffer containing 35 % raffinose (Sigma),
13 1mM PMSF (Sigma), 10 µl of protease inhibitor cocktail (GE Healthcare) and 100 µg of
14 lysostaphin (Sigma). After incubation at 37°C during 30 minutes, surface proteins and
15 protoplasts were separated by low speed centrifugation (2500 x g during 10 minutes). The
16 supernatant was stored at -20°C. Protein concentration was determined using the Bradford
17 reagent in a 96 well plate assay at 595 nm according to the manufacturer's instructions
18 (Sigma). Cell wall samples were precipitated with 2D clean up kit (GE Healthcare) before
19 electrophoresis. 4 µg of proteins from each sample were separated by 1-D electrophoresis.
20 Secreted proteins were prepared according to Ziebandt et al. with minor changes (Ziebandt et
21 al., 2001). The culture was centrifuged at 7000 x g for 10 minutes and the supernatant was
22 filtered through a 0.45 µm filter. The secreted proteins were precipitated with 10 % TCA at
23 4°C overnight. Protein samples were centrifuged at 7000 x g during 1.5 hours at 4°C. Protein
24 pellets were washed three times with ethanol 96%, air-dried during 30 minutes and were
25 resuspended in 150 µL of sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS, 5% DTT, 10%

glycerol and bromophenol blue). An aliquot of 5 μ L of the suspension was submitted to 1-D electrophoresis.

SDS-PAGE and western blotting. Protein extracts were separated by SDS-PAGE on 12% acrylamide separating slab gels (70 X 100 X 0.5 mm), with a 4% acrylamide stacking gel on a miniprotean III gel system (BioRad, Ivry sur Seine, France) according to the conditions described by Laemmli (Laemmli, 1970). Migration was performed for 2 h at room temperature using 80 V which were kept constant. Samples were diluted in 15 μ L of sample buffer and denatured at 100 $^{\circ}$ C for 3 min. Gels were either stained with Coomassie blue R-250 or transferred onto a PVDF membrane (GE Healthcare) by 250 mA at 30 V in Towbin transfer buffer (Towbin et al., 1979) using a Trans-Blot cell (Biorad) for 1.25 hours. Membranes were washed 3 times with Tris Buffered Saline (TBS) pH 7.5 and saturated in blocking solution (3 % non-fat dry milk in TBS with Tween 20 0.3 % (TBS-T)) at 4 $^{\circ}$ C overnight. Membranes were then washed 3 x 10 minutes with TBS-T and incubated with serum (1:40 000 in 25 mL of blocking solution) for 1 hour at room temperature. After washing, membranes were incubated with alkaline phosphatase-conjugated anti-sheep IgG (Sigma) diluted 1:15 000 in 25 ml blocking solution for one hour and finally BCIP/NBT (Sigma) was used to visualize reactive proteins, according to the manufacturer's instructions. Field sera were compared with the mini-protean II Multiscreen apparatus (Biorad). Briefly, after saturation in blocking solution, the membrane was exposed to the different ewe sera (600 μ L of serum diluted 1:40 000 in blocking solution) for 1 hour at room temperature. The following steps were similar to the ones described above.

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1 **Image analysis.** Immuno-developed Western blots were scanned using an Image Scanner II
2 (Amersham biosciences) and further analyzed using ImageQuant 1D software. The number,
3 volume and area of bands were taken into account for the analysis.

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1 **Results and discussion**

2
3 **Choice of culture media and *S. aureus* growth conditions.** Three different culture media
4 were tested in this study. Brain Heart Infusion broth (BHI), which is a rich, non-selective and
5 non-buffered culture medium commonly used for growing *S. aureus* and other bacteria. BHI
6 was used as a control medium in this study.

7 The Roswell Park Memorial Institute medium (RPMI), a synthetic medium for eukaryotic cell
8 culture, which had already been used for culturing *S. aureus* for use in proteomic experiments.
9 Notably, it has been shown that iron depleted RPMI 1640 enhances the production of antigens
10 compared to BHI as determined by SERPA analysis using human sera (Vytvytska et al.,
11 2002). Growth conditions in which there is restriction in the bioavailability of iron can lead to
12 an increase of the expression of virulence factors which are normally expressed *in vivo*
13 (Trivier et al., 1995). The extremely low availability of iron in mammalian interstitial fluid
14 due to the presence of transferrins is an important environmental growth criterion for bacteria.
15 In growth conditions where there is a low concentration of iron and competition for iron with
16 transferrins, *S. aureus* derepresses the genes encoding siderophores, which are low-molecular-
17 mass iron chelators and specific cell wall protein receptors (Modun et al., 1992).

18 Milk and whey have been reported to be media that mimic the growth conditions faced by
19 *Staphylococcus* in mastitis (Lammers et al., 2000). Whey was compared to tryptic soy broth
20 (TSB) and was shown to induce the production of *in vivo*-like antigens (Mamo and Froman,
21 1994b), adhesion of *S. aureus* to bovine mammary epithelial cells (Mamo and Froman,
22 1994a), anti-phagocytic surface properties (Mamo et al., 1991b) and to enhance virulence of
23 *S. aureus* (Mamo et al., 1991a) whereas TSB did not. UF instead of MF was used to study
24 secreted proteins because the presence of milk proteins in MF dramatically impairs secreted
25 protein precipitation.

1 Both RPMI and whey media seem to mimic *in vivo* conditions, however, they have never
2 been compared to each other as culture media to determine which one best mimics the
3 mastitis-like growth conditions.

4 *S. aureus* O46 was cultivated on each of the three media, with and without agitation (Figure
5 1). Both ewe whey and BHI sustained rapid growth of the strain whereas growth on iron-
6 depleted RPMI was significantly slower in both aerobic and anaerobic growth conditions. The
7 calculated growth rates were 0.75 h⁻¹, 0.85 h⁻¹ and 0.62 h⁻¹, for cultivation with agitation on
8 ewe whey, BHI and iron-depleted RPMI, respectively. They were slightly lower (0.58 h⁻¹,
9 0.68 h⁻¹ and 0.59 h⁻¹), for cultivation without agitation. In spite of this, final biomasses did not
10 vary significantly from one medium to another. The time points for culture sample collection
11 in the exponential and stationary phases of growth were determined according to these growth
12 conditions (Figure 1).

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14
15 **Experimental infection and selection of the hyper-immune sera.** A primary lactating ewe
16 was used for bacterial challenge in the two teats with the strain O46. Following the infection,
17 the ewe infected showed a fever (41°C) and polypnea on D2 to D3. The ewe was clinically
18 normal on D4 after the inoculation. The infection caused neither flakes nor clots in the milk
19 and did not induce nodules or induration in the udder. Bacterial shedding began at D1 up to
20 D28 (data not shown). Anti-sheep IgG antiserum was used for Western blot analysis of total
21 lysates to evaluate the IgG reactivity of the sera at different time intervals during experimental
22 infection. Western blotting showed an increased IgG reactivity with serum obtained on D14
23 compared to the other sera collected on D0, D7 and D21. A dilution of 1:40 000 yielded the
24 best signals with only a small background (data not shown). These results corroborate
25 previous SERPA studies using human and bovine sera (Tedeschi et al., 2008; Vytvytska et al.,

1 2002). The considerably large dilution required for immune-revelation with the sera was due
2 to the presence of a wide range of antistaphylococcal antibodies found even in healthy
3 animals (i.e. animals which never suffered from mastitis; (Dryla et al., 2005; Tedeschi et al.,
4 2008). This observation is corroborated in our Western blot experiments: sera from
5 primiparous ewes without mastitis or even from new born lambs recognized some
6 staphylococcal antigens (Figure 4; lanes 7-10).

7 The specificity of the serum obtained was tested against protein samples (total lysates)
8 prepared from *S. aureus*, *Staphylococcus xylosus*, *Lactobacillus helveticus*, *Streptococcus*
9 *thermophilus* and *Escherichia coli* cultures (Figure 2). A dilution of 1/40,000 of the serum
10 obtained at D14 was used to reveal the protein samples. Only 6 proteins were revealed in the
11 *S. xylosus* extract. Two of these proteins co-migrated with *S. aureus* proteins. Such cross-
12 hybridizations may be due to the similarity of some proteins found in the *Staphylococcus*
13 genus. However, the signals generated by these cross-hybridizations were faint. Similarly,
14 faint signals were also obtained with *E. coli* samples (Figure 2). Besides, one cannot exclude
15 that those faint signals were due to previous infections by a staphylococcal and or an *E. coli*
16 strain.

17
18 **Impact of culture medium, growth phase and aeration on immunogenic protein**
19 **production in *S. aureus*.** *S. aureus* protein production is reportedly dependant on i) medium
20 composition (Trivier and Courcol, 1996; Watson and Prideaux, 1979), ii) growth phase of the
21 *S. aureus* culture (Novick, 2003) and iii) oxygen availability (Mayer et al., 1988; Ster et al.,
22 2005). Combinations of these three parameters were tested using protein samples prepared
23 from bacteria harvested at various culture growth phases, and grown in various culture
24 conditions and media. The most relevant results obtained are presented in Figure 3. Among
25 the three media tested, iron-depleted RPMI was the medium where *S. aureus* O46 produced

1 the most numerous immunodetected proteins with up to 22 different immunodetected proteins
2 and the highest average signal volume whatever the growth phase (not shown), culture
3 fractions, or culture conditions analyzed. In the different fractions tested, only a few bands
4 were detected in protein samples prepared from cultures grown on BHI or whey, whereas they
5 were not detected or were much fainter in iron-depleted RPMI samples (Figure 3). Using
6 human sera, Vytvytska et al. demonstrated that cultivation in iron-depleted RPMI induced a
7 greater production of antigenic proteins in *S. aureus* compared to cultivation on BHI. Other
8 works suggested that *S. aureus* cultivation in milk or whey induced the production of *in vivo*-
9 like antigens compared to growth in laboratory rich media. Our study compared for the first
10 time three culture media and showed that, unexpectedly, iron-depleted RPMI better mimics
11 mastitis conditions than do the other two media tested.

12 The expression of numerous genes in *S. aureus* is growth-phase dependant. Notably,
13 the expression of virulence factors is tightly regulated by the accessory gene regulator (*agr*), a
14 central system in virulence regulation based on quorum sensing (Mayer et al., 1988; Novick,
15 2003). In order to determine which growth phase corresponded to the most abundant antigen
16 production, total cell lysate and cell wall protein samples were prepared from cells harvested
17 at different time intervals corresponding to exponential phase, early stationary phase, and late
18 stationary phase. Signals obtained with total cell lysate were more numerous and stronger
19 when protein samples were prepared from exponential compared to early- or late- stationary
20 phase. Cell wall proteins samples prepared from early stationary phase generated more
21 numerous and stronger signals compared to those prepared from log or late stationary phase
22 (not shown). Conversely, the number and strength of western blot signals from secreted
23 proteins (culture supernatant samples) increased from early exponential phase to late
24 stationary phase (not shown). The absence of lactate dehydrogenase activity (a typically
25 intracellular enzyme) in each of the supernatant fractions was demonstrated as shown

1 previously (Hillier and Jago, 1982). Moreover, protein patterns were dramatically different
2 from those of total cell lysate. Together, these results showed that such immunogenic protein
3 accumulation was not due to *S. aureus* cell lysis in late stationary phase. Late stationary phase
4 was thus kept for further analysis of supernatant fraction. These observations can be
5 corroborated with the *agr*-dependant induction of secreted proteins when *S. aureus* culture
6 reaches the post-exponential growth phase (Novick, 2003).

7 Oxygen concentration is an important parameter in *S. aureus* growth and *S. aureus*
8 gene expression varies dramatically between aerobic and anaerobic conditions (Fuchs et al.,
9 2007; Ster et al., 2005). During mastitis, the O₂ concentration in milk decreases dramatically
10 to a level below 10 % of the one found in normal milk (Mayer et al., 1988). In order to test the
11 effect of oxygen on the *in vitro* production of immunogenic proteins, *S. aureus* O46 was
12 grown in whey, BHI and iron-depleted RPMI with or without aeration and protein extraction
13 was carried out during exponential phase and stationary phase. Among the set of conditions
14 tested, cell lysate protein samples prepared from the exponential phase of growth in iron
15 depleted RPMI without agitation resulted in the highest number of immunodetected proteins
16 (19) compared with the other conditions (from 8 in BHI without agitation to 18 in MF without
17 agitation). Band intensity and band area means were higher in samples prepared from iron-
18 depleted RPMI without aeration (Figure 3A). Of note, intensity (or volume) and area of the
19 bands were slightly lower in iron depleted RPMI without aeration compared to the same
20 medium with aeration (0.95 time lower for intensity and 0.92 for area). However, the number
21 of bands present was much higher without aeration (19 versus 13).

22 For cell wall protein extracts, iron depleted RPMI without aeration resulted in the
23 highest number of immunodetected bands. This showed that protein samples from the
24 stationary phase of growth in iron depleted RPMI without aeration enhanced immunoreactive
25 cell wall protein synthesis (Figure 3B). This result is in contradiction with *agr* dependant

1 regulation of surface protein production in *S. aureus*, which is up-regulated during
2 exponential phase and down-regulated during stationary phase. However, *agr*-dependant
3 regulation mostly controls virulence related genes and, in a mastitis context, immunoreactive
4 proteins are not necessarily virulence factors (Tedeschi et al., 2008).

5 Regarding the secreted proteins, protein samples prepared from supernatants of
6 stationary phase of the *S. aureus* culture grown in iron depleted RPMI with aeration resulted
7 in the highest number of detected bands (23) with the highest intensity and band area mean
8 (Figure 3C). Iron depleted RPMI with low oxygen yielded an interesting profile but both the
9 number of bands (22) and the signal intensity (1.4 times lower) were lower. These results
10 were in accordance with the temporal regulation of virulence factor expression by the *agr*
11 system: the expression of genes encoding secreted proteins (e.g. toxins) is activated during the
12 late exponential phase (Novick, 2003). These virulence factors are reportedly highly
13 immunogenic in an infectious context.

14 **Variations in the pattern of *S. aureus* O46 proteins immuno-detected with various ewe**
15 **sera obtained in the field.** The protein patterns of *S. aureus* strains involved in mastitis may
16 vary from one strain to another. Similarly, the immune response of the ewes may vary from
17 an individual to another during mastitis as well as in healthy carriage. As such, ewe sera
18 obtained in the field collected from cases of mastitis involving uncharacterized *S. aureus*
19 strains are likely to generate a variety of immuno-detected protein patterns. To test this
20 hypothesis, protein samples were prepared from *S. aureus* O46 grown in the culture
21 conditions selected in this work. The protein samples were further used for immuno-detection
22 using 10 different ewe sera (Figure 4). Sera were collected from 5 different ewes suffering
23 from diagnosed *S. aureus* mastitis and from 4 healthy animals. The results obtained showed
24 that healthy animals produced antibodies against *S. aureus* (Figure 4, lanes 7 to 10). This can
25 be correlated to the high prevalence of *S. aureus* healthy carriage in sheep flock (Vautor et al.,
26

1 2005). Serum from a new born lamb recognized only 2 proteins with a low intensity. The
2 signal intensity generated by the immunodetection using healthy animal sera was low.
3
4 Overexposition of the immunoblots did not reveal significantly different protein patterns. This
5 suggests that, as opposed to what happens in healthy carriage, the involvement of *S. aureus* in
6 mastitis induces a specific and strong immune response.

7
8 The majority of ewes with mastitis presented high antibody levels against *S. aureus* (Figure 4,
9 lane 1 to 6). One of the sera collected (lane 4) revealed *S. aureus* O46 proteins with a very
10 high intensity. Most of the bands present in profile 1 were also present in the other profiles.
11 Some additional bands were detected in lane 4, especially the ones presenting a low molecular
12 weight. These results showed that immunogenic proteins synthesized during growth in iron
13 depleted RPMI are also recognized by field sera. Experimental infection is a useful approach
14 to identify immunogenic proteins that are recognized by ewe antibodies. However, the use of
15 sera obtained in the field can be a useful tool to validate the immunogenic proteins recognized
16 by animals infected by different strains. Such comparisons might help in identifying and
17 defining a *S. aureus* core seroproteome (i.e. the pool of *S. aureus* proteins common to all *S.*
18 *aureus* strains and recognized by any of the ewe sera tested) and an accessory seroproteome
19 (i.e. the pool of immuno-detected proteins that vary with the *S. aureus* strains and the ewe
20 infected).

21
22 **Conclusion.** SERPA is a promising technique that can be used to investigate immuno-
23 dominant proteins produced by *S. aureus* in a mastitis context, providing that the growth
24 conditions of the *S. aureus* strains used for protein sample preparation mimic as best as
25 possible the environmental conditions of mastitis and induce the expression of the relevant
staphylococcal antigens. In this study, we determined that culturing in iron-depleted RPMI is
the condition that induces the expression and production of the most numerous and immune-

1 responsive staphylococcal antigens, and therefore, the condition that best mimics mastitis.
2 The exponential phase is the ideal growth phase to study antigens present in total cell lysate
3 and early stationary phase is the ideal one to study antigens present in cell wall fractions and
4 in both cases low oxygen enhanced more antigen production. Conversely, late stationary
5 phase in presence of oxygen is the best culture condition to study secreted antigens. The
6 limitation of oxygen appears to be an essential parameter for the mimicking of a mastitis
7 condition. Whey was expected to enhance the *in vivo* expression of antigens but this was not
8 confirmed in this study. However, we used microfiltered whey instead of whey obtained from
9 curd draining after acidification (Mamo et al., 1991a; Mamo et al., 1991b; Mamo and Froman,
10 1994a; Mamo and Froman, 1994b). Variations in whey composition may explain the
11 differences observed here. Growth in whey may induce other kind of non proteinous antigens
12 such as capsular polysaccharides (Sutra et al., 1990) which are also immunogenic (Dryla et
13 al., 2005). The experimental conditions determined in this study are crucial to further develop
14 a SERPA approach for the identification of *S. aureus* antigens involved in the sheep immune
15 response during mastitis.

16
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1 **Figure Legends**

2 **Figure 1.** Growth kinetics of *S. aureus* O46 in BHI (Δ , upper panel), MF (\diamond , middle panel),
3 and RPMI+iron chelator (\square , lower panel) at 37°C with (dotted lines) or without (plain lines)
4 agitation. Data are the average of three independent experiments. Arrows indicate the time
5 points when samples were harvested for proteomic analysis.

6
7 **Figure 2.** Western blot analysis of *S. aureus* O46 total cell lysates after growth in iron
8 depleted RPMI without agitation (1), a strain of *Staphylococcus xylosus* (2), *Lactobacillus*
9 *helveticus* (3), *Streptococcus thermophilus* (4), *E. coli* (5) using D14 diluted to 1:40 000 ewe
10 serum. Migration positions of size standards are presented on the right side of the figure.

11
12 **Figure 3.** Western blot (left panels) and image analysis (right panels) of total lysates (A), cell
13 wall extracts (B) and secreted proteins (C) of *S. aureus* O46 using D14 diluted 1:40 000 ewe
14 serum. Bacteria were grown in microfiltered milk (MF or UF), Brain Heart Infusion (BHI), or
15 RPMI depleted of iron (RPMI iron -) either with aeration (+) or without aeration (-) and either
16 to exponential phase (A), to early stationary phase (B), or to stationary phase (C). Left panels:
17 Migration positions of size standards and bands detected by image analysis are presented on
18 the left and right side of Western blots, respectively. Right panels: histograms present the
19 number of bands (dashed), band volume (white), and band area (grey) means. Volume and
20 area are expressed in arbitrary units given by the Image Quant 1D software.

21
22 **Figure 4.** Western blot analysis of total lysate proteins of *S. aureus* O46 grown in iron
23 depleted RPMI with low oxygen using D14 ewe serum (1), primiparous lacaune ewe serum
24 with clinical mastitis (2), multiparous ewe serum with subclinical mastitis (3), multiparous
25 ewe with subclinical mastitis (4), primiparous ewe serum with clinical mastitis (5),

1 multiparous ewe with clinical mastitis (6), 3 days lamb serum (7), primiparous lactating
2 lacaune ewe with no mastitis (8), new born lamb serum (9), primiparous lactating lacaune
3 ewe with no mastitis (10). All sera were diluted to 1:40 000. Migration positions of size
4 standards and bands detected by image analysis are presented on the left and right side of
5 Western blots, respectively. Number of bands, (dashed) band volume (white) and band area
6 (grey) means are presented on the graph. Volume and area are expressed in arbitrary units
7 given by the Image Quant 1D software.

8

Figure(s)

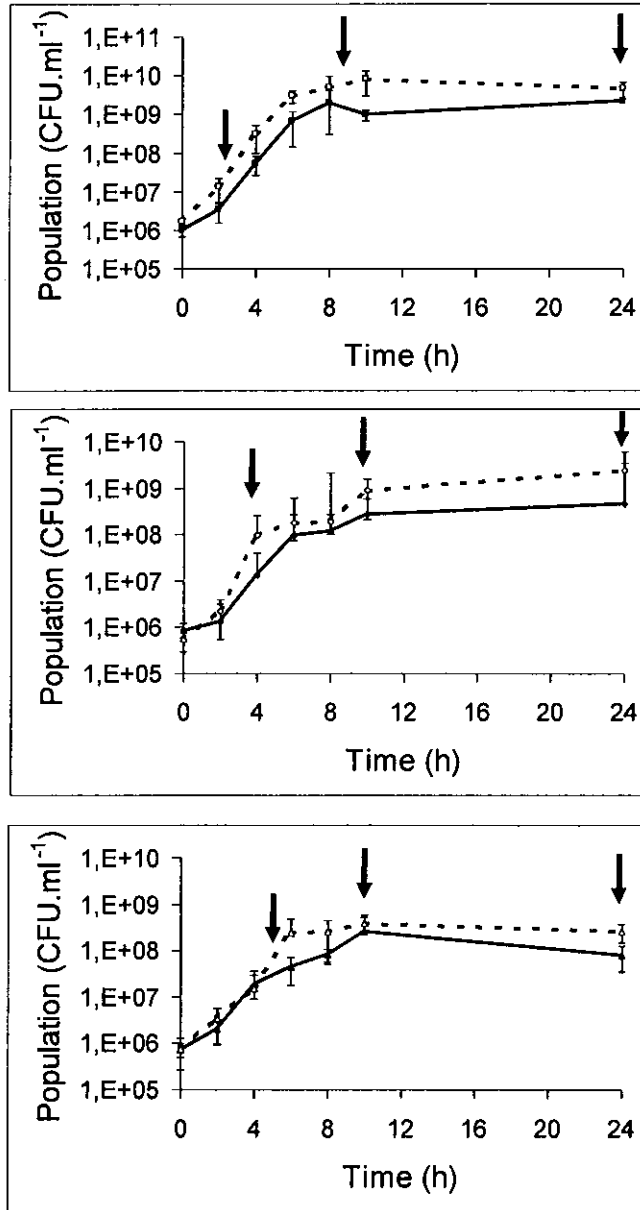


Figure 1

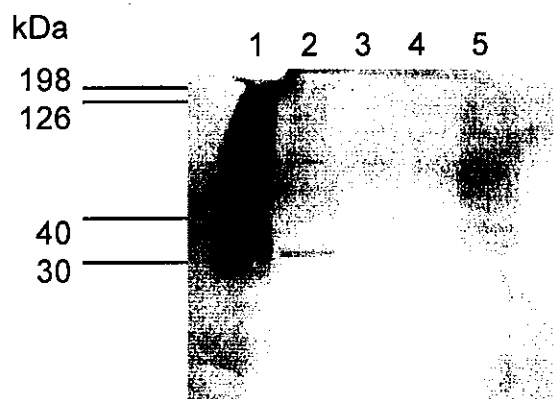


Figure 2

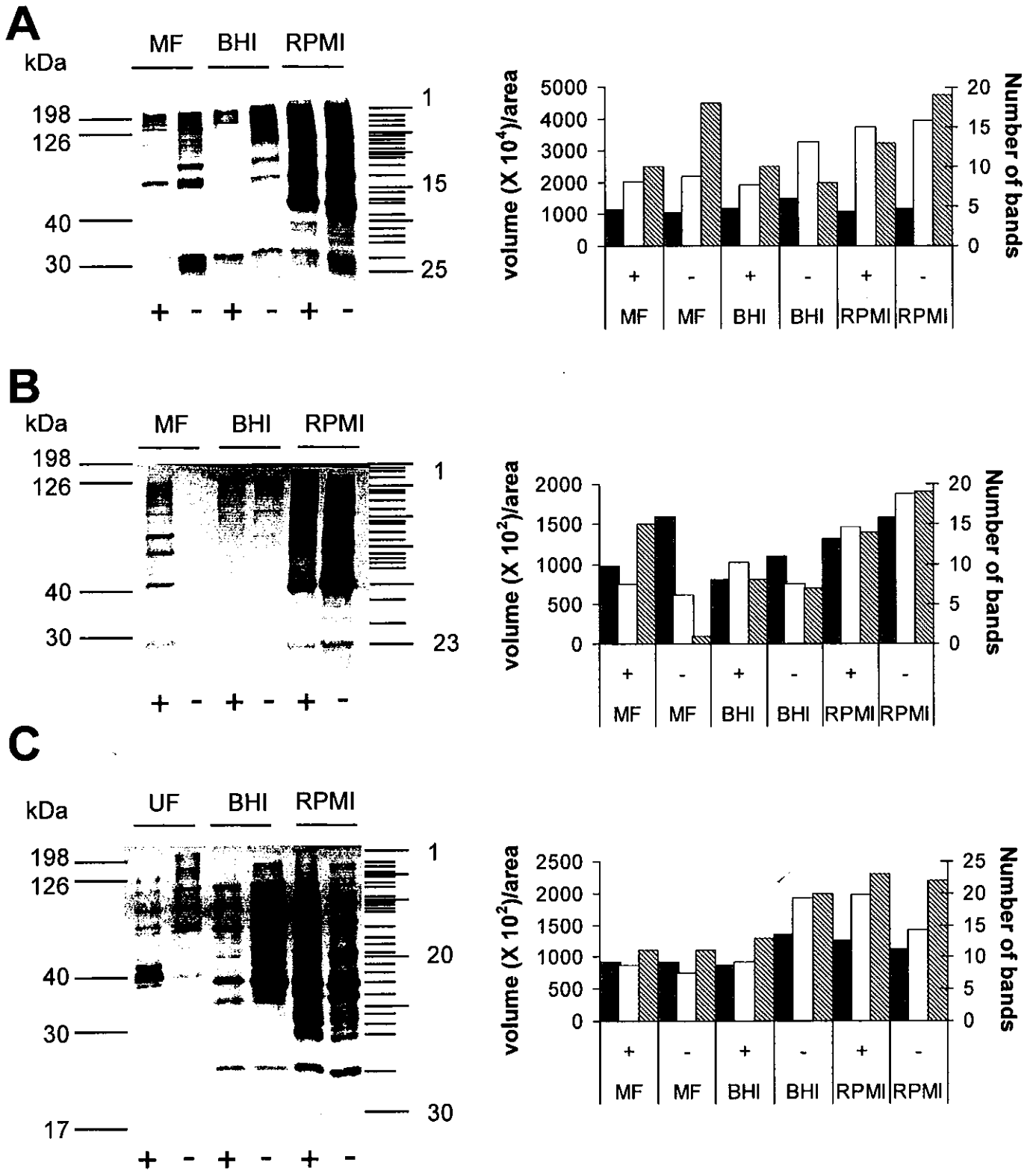


Figure 3

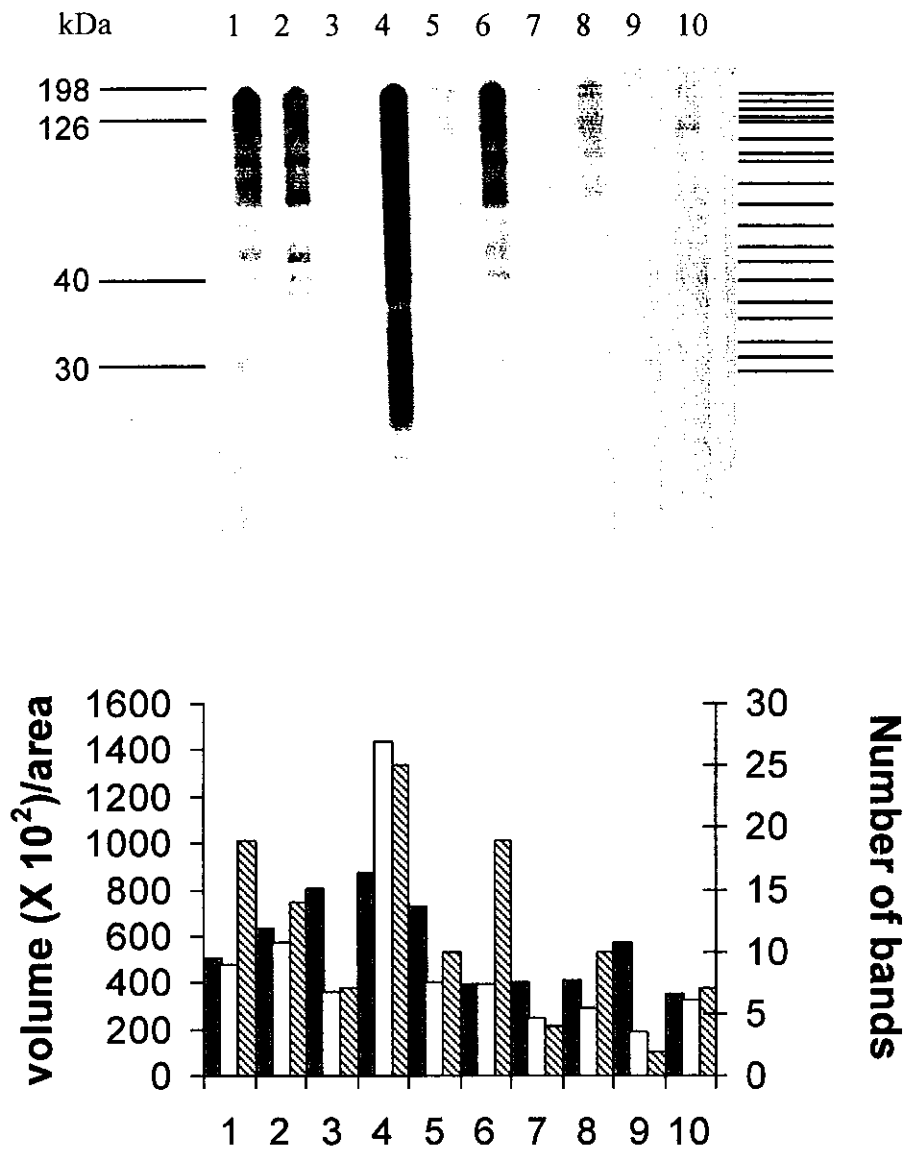


Figure 4