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## Short communication

***Staphylococcus aureus* proteins differentially produced in ewe gangrenous mastitis or ewe milk**

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## ABSTRACT

Despite being one of the main pathogens involved in ruminant mastitis, little is known about what proteins *Staphylococcus aureus* does express, *in vivo*, during the infection. Here, two *S. aureus* strains were isolated from curds formed within the udder of two ewes suffering from gangrenous mastitis. Protein samples were prepared from cell fractions and were analyzed using 1D-LC MS/MS. Results were compared to 1D-LC MS/MS analysis of the same *S. aureus* strains grown in ewe milk. A total of 365 proteins were identified. Most of them were related to cellular metabolism, cellular division and stress response. Half of the proteins were found in both conditions but a substantial number were specifically found in *in vivo* conditions and gave indications about the active metabolic status and the stresses encountered by *S. aureus* within the cistern during a gangrenous mastitis.

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## 1. Introduction

Despite great research efforts, mastitis remains a major concern for dairy ruminant herds worldwide. It causes huge economic losses and affects animal health as well as milk quality (Le Maréchal et al., 2011c). Investigation of *Staphylococcus aureus* transcriptome or proteome *in vivo* stumbles on technical bottlenecks such as the difficulty to localize the infection site within the udder and the low amounts of *S. aureus* cells that can be recovered from such infections. Serological proteome analysis (SERPA) was

recently used to circumvent these difficulties (Le Maréchal et al., 2011d; Tedeschi et al., 2009). It allows investigating the *S. aureus* proteins recognized by the host's immune response and thus identifying proteins that are indeed produced by *S. aureus* during mastitis. However, such identification is restricted to the immune reactive proteins and does not shed light on other non immune reactive proteins that can nevertheless be produced during infection. Recent advances in proteomics (namely, GeLC-MS/MS) allow discovering proteins from complex samples containing low amounts of proteins. The objective of this work was to identify the proteins that are differentially produced in bacterial cells or tightly cell-bound when *S. aureus* grows in gangrenous mastitis conditions compared to growth in the same milk medium but in laboratory conditions. A better inventory of the proteins produced by *S. aureus*, strains whatever their degree of virulence during the mastitis onset may indeed help understanding pathways important for mastitis pathogenesis.

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## 2. Materials and methods

### 2.1. Bacterial strains, growth conditions and protein extraction

*S. aureus* strains O11 and O46 are two clonally related strains originally isolated from gangrenous and subclinical ewe mastitis cases, respectively (Vautor et al., 2009). *In vivo* samples analyzed in the present study were obtained from animals experimentally infected in an *S. aureus* challenge study conducted at the French National Agency for Food, Environmental and Occupational Health and Safety (Le Maréchal et al., 2011d) and according to the Regional Committee for Animal Use and Care (Côte d'Azur, France), recorded under reference NCA/2008-14/12-09n. In this previous experiment, two ewes developed a gangrenous mastitis (one after being inoculated by strain O11 and one by strain O46) shortly after infection. Such sudden onset of a severe infection sometimes occurs in small ruminants (Cetin et al., 2005; Le Maréchal et al., 2011d; Rainard, 2007) and the two suffering ewes were humanely euthanized 24 h post-infection. Coagulated milk was then aseptically collected from the cistern and stored at  $-80^{\circ}\text{C}$ . Bacteria extraction was performed as previously described (Cretenet et al., 2011). Briefly, to harvest bacteria, 10 g of *in situ* coagulated milk were homogenized with 90 mL of trisodium citrate solution (2% (w/v)) at  $4^{\circ}\text{C}$  using a mechanical Waring blender. Three 30-s mixing sequences were performed at high speed, and three 10-s mixing sequences performed at low speed. Cells from 10 mL of suspension were then recovered by centrifugation for 5 min at  $6000 \times g$  at  $4^{\circ}\text{C}$ . These steps allowed removing most of the milk proteins, which abundance could interfere with the identification of bacterial proteins. *In vitro* samples were obtained after incubation of an overnight culture of strain O11 and strain O46 in BHI diluted 1:1000 in 100 mL of semi-skimmed ewe milk at  $37^{\circ}\text{C}$  without agitation during 24 h (the same time lapse as in the ewe mammary gland). Bacteria were harvested as described above. After washing in ice-cold PBS, total lysates were prepared using lysostaphin treatment and sonication. The lysates were centrifuged to obtain the bacterial protein extracts (Le Maréchal et al., 2009).

### 2.2. GelC-MS/MS analysis

Protein extracts were separated by SDS-PAGE on 12% acrylamide separating slab gels with a 4% acrylamide stacking gel on a Protean II gel system (BioRad, Ivry sur Seine, France). Migration was performed overnight at room temperature under a constant 60 V voltage. Samples were diluted in 45  $\mu\text{L}$  of sample buffer and denatured at  $100^{\circ}\text{C}$  for 3 min. Gels were stained with Coomassie blue R-250. Each lane was manually cut into slices and transferred into 0.5 mL eppendorfs. After washing with acetonitrile, proteins were reduced (10 mM DTT), then alkylated (55 mM iodoacetamide) and finally dried under vacuum in a SpeedVac concentrator (SVC100H-200; Savant, Thermo Fisher Scientific, Waltham, MA, USA). In-gel trypsin digestion was performed overnight at  $37^{\circ}\text{C}$  and

stopped with spectrophotometric-grade trifluoroacetic acid (TFA) (Sigma–Aldrich).

Nano-LC experiments were performed on the supernatants using an on-line liquid chromatography tandem mass spectrometry (MS/MS) setup using a Dionex U3000-RSLC nano-LC system fitted to a QSTAR XL (MDS SCIEX, Ontario, Canada) equipped with a micro-electrospray ion source (ESI) (Proxeon Biosystems A/S, Odense, Denmark). Samples were first concentrated on a PepMap 100 reverse-phase column (C18, 5  $\mu\text{m}$ , 300- $\mu\text{m}$  inner diameter (i.d.) by 5 mm length) (Dionex, Amsterdam, The Netherlands). Peptides were separated on a reverse phase PepMap column (C18, 3  $\mu\text{m}$ , 75  $\mu\text{m}$  i.d. by 150 mm length) (Dionex) at  $35^{\circ}\text{C}$ , using solvent A (2% (vol/vol) acetonitrile, 0.08% (vol/vol) formic acid, and 0.01% (vol/vol) TFA in deionized water) and solvent B (95% (vol/vol) acetonitrile, 0.08% (vol/vol) formic acid, and 0.01% (vol/vol) TFA in deionized water). A linear gradient from 10 to 40% of solvent B in 45 min was applied for the elution at a flow rate of 0.3  $\mu\text{L}/\text{min}$ . Eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode. A full continuous MS scan was carried out followed by three data-dependent MS/MS scans. Spectra were collected in the selected mass range 400–2000 m/z for MS and 60–2000 m/z for MS/MS spectra. The three most intense ions from the MS scan were selected individually for collision-induced dissociation (1+ to 4+ charged ions were considered for the MS/MS analysis). The mass spectrometer was operated in data-dependent mode automatically switching between MS and MS/MS acquisition using Analyst QS 1.1 software. The instrument was calibrated by multipoint calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from  $\beta$ -casein,  $\beta$ -CN (193-209). The proteins present in the samples were identified from MS and MS/MS data using MASCOT v.2.2 software for search into two concatenated databases: (i) a homemade database containing all the predicted proteins of the *S. aureus* strains O11 and O46 used in this study and (ii) a portion of the UniProtKB database corresponding to the *S. aureus* taxonomic group. Search parameters were set as follows. A trypsin enzyme cleavage was used, the peptide mass tolerance was set to 0.2 Da for both MS and MS/MS spectra, and two variable modifications (oxidation of methionine and deamidation of asparagine and glutamine residues) were selected. A maximum of one missed tryptic cleavage site was allowed. For each protein identified in NanoLC-ESI-MS/MS, a minimum of two peptides with MASCOT score corresponding to a *p*-value below 0.05 were necessary for validation of the protein. For automatic validation of the peptides from MASCOT search results, the 1.19.2 version of the IRMa software was used (Dupierris et al., 2009).

## 3. Results and discussion

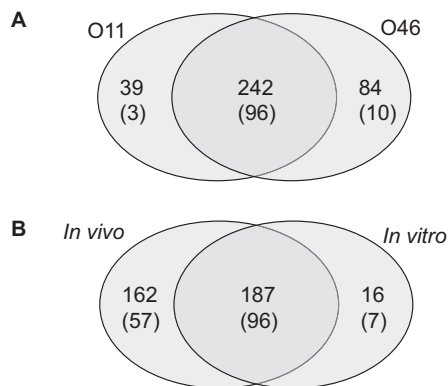
*In situ* study of pathogens is tricky but really informative to better understand the infectious processes. In contrast to *Escherichia coli* mastitis, severity of *S. aureus* mastitis depends on strain-specific rather than on host-specific characteristics (Burvenich et al., 2003; Haveri et al.,

2007; Le Maréchal et al., 2011b). However, in some cases, the health status of individual animals (e.g. transient weakness, immunodeficiency, other infection, etc.) may lead to the onset of severe symptoms even when the infection involves an *S. aureus* strain like O46 that reportedly induces mild mastitis (Le Maréchal et al., 2011d). The two mastitis cases used in this work were two severe gangrenous mastitis cases with a sudden onset (24 h post-infection), each of which resulted from an experimental infection with two *S. aureus* strains. Investigating the proteome of these two different strains might help discovering what *S. aureus* has to cope with during the onset of a gangrenous mastitis, beyond any strain specific features. Here, we report the first proteome analysis of bacteria extracted from curds resulting from the intramammary coagulation of cisternal milk after gangrenous mastitis. Using 1D-LC MS/MS, we managed to identify 365 proteins, which were produced during gangrenous ewe mastitis by strain O11 or O46, two *S. aureus* strains isolated from mastitis and fully characterized (Le Maréchal et al., 2011a, 2011b, 2011d) (Table S1, supplemental data).

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2013.01.013>.

Substantial differences were observed when considering each of the two strains. A pool of 242 proteins was found common to strain O11 and O46 grown *in vitro* or *in vivo*, whereas 39 were strain O11-specific, and 80 were strain O46-specific (Fig. 1A). Some strain O11-specific proteins identified here were previously shown overproduced in O11 (e.g. LukM and LukF-PV), compared to strain O46 (Le Maréchal et al., 2011b). On the other hand, the gene encoding SpoVG, a strain O46-specific protein, was previously shown to be truncated in strain O11 (Le Maréchal et al., 2011b).

The most apparent differences were observed when considering the *in vitro* or *in vivo* growth conditions. Under both growth conditions 187 proteins were produced, in



**Fig. 1.** (A) Venn diagram summarizing strain O11- and O46-specific proteins. Some proteins were found in strain O11 or O46 samples, only. In between parentheses, the number of proteins found common in both *in vitro* and *in vivo* conditions. (B) Venn diagram summarizing the gangrenous mastitis- and milk-specific *S. aureus* proteins. In total, 365 proteins were identified, some of which were found in gangrenous mastitis samples or milk samples, only. In between parentheses, the number of proteins found common in both O11 and O46 strains.

one strain or the other (Fig. 1B). Ninety six of these proteins (~31%) were identified in both *in vivo* and *in vitro* samples and in both strains. They include numerous proteins involved in central metabolism and pathways that are likely necessary to support bacterial growth whatever the conditions. Some proteins, such as LacD, LacE, LacG and Ldh reflect adaptation to the milk environment found in both growth conditions (Table S1).

Of 365 proteins in total identified under either or both growth conditions, 35 belong to the set of 89 proteins that were previously shown to be produced during mastitis using serological proteome analysis (Table 1). A majority (32 proteins) was previously found immunoreactive in both strains (core seroproteome) whereas 3 were strain O11-specific (accessory proteome). Production of LukF'-PV was confirmed to be produced by strain O11 only. This protein has been reported to be associated with strains isolated from gangrenous mastitis in small ruminants (Le Maréchal et al., 2011d; Rainard, 2007). However, it appears here that O46, for which no production of LukF'-PV was detected, can nevertheless induce a gangrenous mastitis suggesting that other factors (likely host factors, here) are involved in the sudden onset of severe mastitis. On the contrary, we were not able to detect other well characterized virulence factors, which were previously shown to be produced during mastitis (Le Maréchal et al., 2011d). These proteins might have been eliminated during samples preparation since, to minimize contamination with milk proteins, cell pellets were washed prior to protein extraction.

Among the 35 reportedly immune reactive proteins identified, 20 proteins (57%) were identified on the basis of a high number of peptides (above 10 peptides) with 10 proteins being identified on the basis of more than 20 peptides, namely, bifunctional acetaldehyde-CoA/alcohol dehydrogenase (42 and 39 peptides for strain O11 and O46, respectively, in gangrenous curd), formate acetyltransferase (36/41), Fda (21/22), enolase (22/24), elongation factor Tsf (21/20), and elongation factor G Fus (28/24), RpoB (27/40), RpoC (41/53), ClpC (17/23), and probable malate:quinone oxidoreductase 1 (19/20).

Some of these proteins are known to be moonlighting proteins, i.e. they have different functions depending on their localization. For example, GAPDH and enolase have been shown to play a role in adhesion when surface exposed (Antikainen et al., 2007; Pancholi and Chhatwal, 2003).

Most proteins identified in *S. aureus* strains isolated from the gangrenous curd belong to functional categories in relation with cellular metabolism (Fig. 2). The presence of RpoB and RpoC (two RNA polymerases; Table 1) suggests an active metabolism of *S. aureus* within the mammary gland during acute infection. Proteins involved in cell division were also present (FtsZ, EzrA), which may also indicate bacterial multiplication within the curd.

Besides, several stress related proteins were also identified, including chaperones such as DnaK, GroEL, Clp proteins, detoxifying enzymes, such as superoxide dismutases (SodA and SodM), reductases (AhpC, AhpF and TrxB), UreE, and putative universal stress protein, showing that the conditions encountered by *S. aureus* in the

**Table 1**  
Proteins identified in this study that were previously shown immunoreactive.

Description <sup>a</sup>	Accession <sup>b</sup>	Gene name <sup>c</sup>	O11 <sup>d</sup>	O46 <sup>e</sup>	SERPA <sup>f</sup>	No. of peptides O11 curd <sup>g</sup>	No. of peptides O46 curd <sup>h</sup>	No. of peptides O11 milk <sup>i</sup>	No. of peptides O46 milk <sup>j</sup>
<b>Energy production and conversion</b>									
L-lactate dehydrogenase 1	F0D325_STAAU	ldh	SAO11_0603	SAO46_0550	X (O11)	13	13	6	9
Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	F0D3T4_STAAU		SAO11_0863	SAO46_0494	X (core)	42	39	16	34
Formate acetyltransferase	F0D407_STAAU		SAO11_0936	SAO46_0593	X (core)	36	41	26	35
<b>Cell cycle control and mitosis</b>									
Cell division protein ftsZ	F0D1E9_STAAU	ftsZ	SAO11_0025	SAO46_1757		12	7	8	14
Septation ring formation regulator EzrA	F0D2F9_STAAU	ezrA	SAO11_0386	SAO46_1105		16	11	4	12
<b>Nucleotide metabolism and transport</b>									
Adenylate kinase	F0D4J7_STAAU	adk	SAO11_1227	SAO46_1328	X (core)	2	3		1
Purine nucleoside phosphorylase deoD-type 2	F0D6F7_STAAU	deoD	SAO11_1788	SAO46_1651	X (core)	2	3	2	2
Inosine-5'-monophosphate dehydrogenase	F0D6Q0_STAAU	guaB	SAO11_1881	SAO46_1449	X (core)	18	19	10	15
<b>Carbohydrate metabolism and transport</b>									
Fructose-diphosphate aldolase class 1	F0D320_STAAU	fda	SAO11_0598	SAO46_0554	X (core)	21	22	12	20
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	F0D5Q2_STAAU	gpmA	SAO11_1532	SAO46_1391	X (core)	5	12		1
Glyceraldehyde-3-phosphate dehydrogenase	F0D8I7_STAAU	gap	SAO11_2520	SAO46_2072	X (core)	17	16	9	14
Triosephosphate isomerase	F0DF58_STAAU	tpiA	SAO11_2522	SAO46_2074	X (core)	11	12	6	8
Enolase	F0D8J1_STAAU	eno	SAO11_2524	SAO46_2076	X (core)	22	24	18	19
<b>Lipid metabolism</b>									
(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase	F0D6M2_STAAU	fabZ	SAO11_1853	SAO46_1611	X (core)	2			
Acetoin reductase	F0D3K9_STAAU		SAO11_0787	SAO46_0963	X (core)	10	10	6	6
<b>Translation</b>									
Elongation factor Ts	F0D1M7_STAAU	tsf	SAO11_0103	SAO46_0015	X (core)	21	20	9	16
30S ribosomal protein S4	F0D2F7_STAAU	rpsD	SAO11_0384	SAO46_1103	X (core)	14	10	7	5
Elongation factor P	F0D3E4_STAAU	efp	SAO11_0722	SAO46_0849	X (core)	3	4		
50S ribosomal protein L3	F0D4H6_STAAU	rplC	SAO11_1206	SAO46_1307	X (core)	7	2	5	7
Elongation factor G	F0D596_STAAU	fus	SAO11_1375	SAO46_0988	X (core)	28	24	15	31
Elongation factor Tu	F0D597_STAAU	tuf	SAO11_1376	SAO46_0987	X (core)	19	15	16	20
30S ribosomal protein S1	F0D625_STAAU	rp	SAO11_1656	SAO46_1856	X (core)	19	14	12	16
50S ribosomal protein L25	F0D893_STAAU	rplY	SAO11_2426	SAO46_2014	X (core)	5	6	5	4
<b>Transcription</b>									
GTP-sensing transcriptional pleiotropic repressor CodY	F0D1M4_STAAU	codY	SAO11_0100	SAO46_0012		12	11	4	7
DNA-directed RNA polymerase subunit beta	F0D591_STAAU	rpoB	SAO11_1370	SAO46_0993		27	40	5	24
DNA-directed RNA polymerase subunit β'	F0D592_STAAU	rpoC	SAO11_1371	SAO46_0992		41	53	1	47
<b>Replication and repair</b>									
DNA polymerase III subunit β	F0D5D0_STAAU	dnaN	SAO11_1409	SAO46_0743	X (core)	7	14	1	6
<b>Cell wall/membrane/envelop biogenesis</b>									
Putative septation protein spoVG	F0D890_STAAU	spoVG	SAO11_2423	SAO46_2017			2		
<b>Post-translational modification, protein turnover, chaperone functions</b>									
Putative ATP-dependent protease protein	F0DA01_STAAU	cipB	SAO11_0300	SAO46_0263		7	7		
Trigger factor	F0D2K1_STAAU	tig	SAO11_0428	SAO46_1149	X (core)	14	16	12	15
ATP-dependent proteinase chain	F0D379_STAAU	clpL	SAO11_0657	SAO46_1215		4	14	4	18
Urease accessory protein ureE	F0DAE9_STAAU	ureE	SAO11_1165	SAO46_0411			3		
60 kDa chaperonin	F0D531_STAAU	groEL	SAO11_1310	SAO46_1078		19	18	1	3
Endopeptidase	F0D573_STAAU	clpC	SAO11_1352	SAO46_1011		17	23		
Alkyl hydroperoxide reductase subunit C	F0D6Q8_STAAU	ahpC	SAO11_1889	SAO46_1458	X (core)	2	5	4	2
Alkyl hydroperoxide reductase subunit F	F0DDE3_STAAU	ahpF	SAO11_1890	SAO46_1459	X (O11)		8		4

Table 1 (Continued)

Description <sup>a</sup>	Accession <sup>b</sup>	Gene name <sup>c</sup>	O11 <sup>d</sup>	O46 <sup>e</sup>	SERPA <sup>f</sup>	No. of peptides O11 curd <sup>g</sup>	No. of peptides O46 curd <sup>h</sup>	No. of peptides O11 milk <sup>i</sup>	No. of peptides O46 milk <sup>j</sup>
Thioredoxin reductase	F0D8B3_STAAU	trxB	SAO11_2446	SAO46_2742	X (O11)	6	8		5
Molecular chaperone DnaK (Fragment)	F0D8Q4_STAAU	dnaK	SAO11_2587	SAO46_2761	X (core)	7	6	3	3
ATP-dependent Clp protease proteolytic subunit	F0D8I2_STAAU	clpP	SAO11_2515	SAO46_2067			3	1	
<b>Inorganic ion transport and metabolism</b>									
Superoxide dismutase	F0D3G9_STAAU	sodA	SAO11_0747	SAO46_0874		2	10	2	2
Superoxide dismutase	F0D3K3_STAAU	sodM	SAO11_0781	SAO46_0969		4	8	2	
Iron-repressed lipoprotein	F0D698_STAAU	mntC	SAO11_1707	SAO46_1927	X (core)	17	11		4
General functional prediction only									
Probable malate:quinone oxidoreductase 1	F0D319_STAAU	mgo	SAO11_0597	SAO46_0330	X (core)	19	20	7	13
Alcohol dehydrogenase	F0D6C2_STAAU	adhA	SAO11_1731	SAO46_1951	X (core)	17	16	11	14
<b>Signal transduction</b>									
Putative universal stress protein	F0D2H1_STAAU		SAO11_0398	SAO46_1117	X (core)	3		1	
Respiratory response protein, SrrA	F0D610_STAAU	srrA	SAO11_1641	SAO46_2509		5	4		
<b>Virulence-defense mechanism</b>									
Leukocidin chain lukM	F0D7A7_STAAU		SAO11_2088	SAO46_2661	X (core)	2			
Panton-Valentine leukocidin LukF-PV chain	F0D7A8_STAAU		SAO11_2089	SAO46_2662	X (core)	2			
<b>Unknown function</b>									
Ribosomal subunit interface protein (fragment)	F0D7N5_STAAU	yfiA	SAO11_2217	SAO46_2753	X (core)	3	5	2	3
Peptidyl-prolyl cis-isomerase (fragment)	F0D8V0_STAAU		SAO11_2633	SAO46_2695	X (core)	3			
Alkaline shock protein 23	F0D8G3_STAAU	asp23	SAO11_2496	SAO46_2306		5	5		

<sup>a</sup> Proteins are classified in Gene Ontology functional classes. Names are given according to annotation of available *S. aureus* sequence genomes.

<sup>b</sup> Accession number on Uniprot.

<sup>c</sup> Gene name.

<sup>d</sup> Coding sequence numbers corresponding to the identified proteins in *S. aureus* O11.

<sup>e</sup> Coding sequence numbers corresponding to the identified proteins in *S. aureus* O46.

<sup>f</sup> Also identified in Le Maréchal et al. (2011d). Proteins belonging to the core seroproteome (core) and O11 accessory seroproteome (O11) are indicated.

<sup>g</sup> Number of peptides identified in sample from curd isolated after infection by O11.

<sup>h</sup> Number of peptides identified in sample from curd isolated after infection by O46.

<sup>i</sup> Number of peptides identified in sample from O11 grown *in vitro* in ewe milk.

<sup>j</sup> Number of peptides identified in sample from O46 grown *in vitro* in ewe milk.

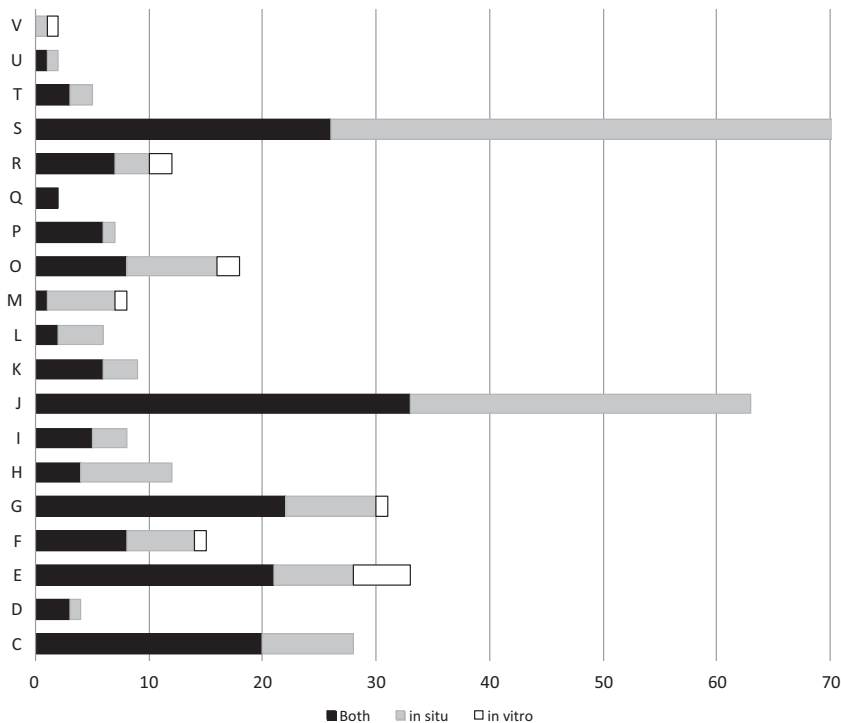


Fig. 2. Number of proteins identified in this study per functional categories. Proteins found in both conditions (milk and gangrenous curd) are in black, found only in the gangrenous curd are in grey and only found in milk are in white. C: Energy production and conversion, D: cell cycle control and mitosis, E: amino acid metabolism and transport, F: nucleotide metabolism and transport, G: carbohydrate metabolism and transport, H: coenzyme metabolism, I: lipid metabolism, J: translation, K: transcription, L: replication and repair, M: cell wall/membrane/envelop biogenesis, O: post-translational modification, protein turnover, chaperone functions, P: inorganic ion transport and metabolism, Q: secondary structure, R: general functional prediction only, S: function unknown, T: signal transduction, U: intracellular trafficking and secretion, and V: virulence and defense mechanism. See Table S1 for details.

gangrenous mastitis context are somehow stressful. Several regulators which expression have been shown to be linked to environmental conditions were also detected. SpoVG, SarA and Asp23 are part of sigmaB regulon (Bischoff et al., 2004; Chan et al., 1998; Kullik et al., 1998; Kullik and Giachino, 1997). SigmaB is involved in the regulation of many cellular processes, including stress responses, intermediary metabolism and virulence and is induced under environmental stresses (Bischoff et al., 2004; Chan et al., 1998). It is well-known that oxygen level is very low in the mammary gland especially during mastitis (Mayer et al., 1988). SrrA, which plays a role in gene regulation under anaerobic conditions (Fuchs et al., 2007), was detected in *in vivo* samples. Its production may be linked to low oxygen conditions found during mastitis. Finally, we also detected CodY in *in vivo* samples, a regulator involved in the adaptation response to starvation (Stenz et al., 2011). Detection of these regulators is particularly informative for understanding infection process. They are indeed indicators of the stressful conditions encountered in gangrenous mastitis context, which include low oxygen availability and starvation. Besides, these regulators are involved in the regulation of expression of virulence factors which may have a preponderant role in symptoms observed during gangrenous mastitis.

Milk or whey is currently considered the best media to mimic the conditions bacteria encounter in the first steps

of intramammary infection (Lammers et al., 2000). Growth in milk or whey was shown to induce the production of anti-phagocytic surface properties (Mamo et al., 1991b) and *in vivo*-like antigens (Mamo and Froman, 1994b), the virulence of *S. aureus* (Mamo et al., 1991a), and the adhesion of *S. aureus* to bovine mammary epithelial cells (Mamo and Froman, 1994a). However, we previously showed that deferroxamine-RPMI (a cell culture medium) could be considered as a good model medium as well (Le Maréchal et al., 2009). In this study, curd was sampled from the mammary gland of ewes. Ewe milk was thus thought to be more relevant than RPMI to be used as an *in vitro* reference medium. Growth in milk allowed the induction of most of the same metabolic pathways and some of the stress-induced proteins as illustrated in Fig. 2, but did not totally mimic conditions found in the udder cistern. In particular, higher numbers of proteins were found in *S. aureus* grown under *in vivo* conditions compared to bacteria grown under *in vitro* conditions. These proteins were mostly involved in replication and repair (4 proteins were identified in *in vivo* and not *in vitro* growth conditions out of the 6 herein identified proteins belonging to this Gene Ontology Functional class), signal transduction (2 proteins identified in *in vivo* and not *in vitro* growth conditions out of 5 herein identified proteins belonging to this Gene Ontology Functional class) and post-translational modifications (9 proteins identified in *in*

*vivo* and not *in vitro* growth conditions out of 17 herein identified proteins belonging to this Gene Ontology Functional class) (Table S1). These observations suggest that bacteria are actively multiplying in the cistern, but not in milk 24 h post infection and post inoculation. The differences observed might result from different growth rates in the *in vivo* and the *in vitro* conditions and/or from the absence of immune response, which might trigger *S. aureus* responses when growing under *in vivo* conditions. They however reflect that growth in milk without agitation does not completely mimic cistern conditions.

Altogether these results give information about what *S. aureus* can produce during gangrenous mastitis. Most proteins that were found differentially produced in gangrenous context versus laboratory conditions were found common to both strains. As results presented here are based on a single *in vivo* experiment, one might keep in mind that some of the proteins identified here were strain-specific and that, by extension, different proteomic profiles (especially in terms of accessory proteome) may result from similar experiments with other *S. aureus* mastitis isolates. Recent studies attempted to identify pathogens' proteome *in vivo* in animal or human pathogens (Hughes et al., 2007; Kruh et al., 2010; Kuntumalla et al., 2011; Sengupta and Alam, 2011). *S. aureus* cell fractions were analyzed here and thus many of the secreted virulence factors known to be involved in pathogenesis could not be detected. A majority of the proteins identified was assigned to functional categories such as energy production and conversion, amino acid metabolism and transport, carbohydrate metabolism and transport, and translation. Their presence in mastitis samples showed that *S. aureus* is metabolically active during acute mastitis and has to tackle stress, starvation and low oxygen. Growth in milk with no aeration during 24 h seems to partially mimic *in vivo* conditions but is not fully satisfactory.

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