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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 cellbound 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 °C. Bacteria extraction was performed as previously described (Cretenet et al., 2011). Briefly, to harvest bacteria, 10g of in situ coagulated milk were homogenized with 90 mL of trisodium citrate solution (2% (w/v)) at 4 °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 °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 °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 μ L of sample buffer and denatured at 100 °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 °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 reversephase column (C18, 5 µm, 300-µm inner diameter (i.d.) by 5 mm length) (Dionex, Amsterdam, The Netherlands). Peptides were separated on a reverse phase PepMap column (C18, $3 \mu m$, $75 \mu m$ i.d. by 150 mm length) (Dionex) at 35 °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 µL/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 β -casein, β -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 hostspecific 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 $(\sim 31\%)$ 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.

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Trissephosphate isomerase FDDF38_STANU tpi/ SA046_2076 X (core) 11 12 6 8 Enolase FDDF38_STANU eno SA011_2524 SA046_2076 X (core) 22 24 18 19 Enolase FDDF38_STANU eno SA011_2524 SA046_2076 X (core) 22 24 18 19 (3R)-Hydroxymyristoyl-Jacyl- FDD6M2_STANU eno SA011_1232 SA046_0063 X (core) 10 6 6 Carrier-proteinj dehydratae FDDTM7_STANU tsf SA011_0103 SA046_0015 X (core) 14 10 7 5 Zos ribosomal protein 54 FDDTM7_STANU tsf SA011_0103 SA046_0015 X (core) 3 4 50 10 7 5 7 Elongation factor 7s FDDTM7_STANU tsf SA011_0103 SA046_0048 X (core) 3 4 50 10 7 5 7 10 10 7 5 50 5 7 10 50 10 50 10 50 10 <td>Clyceraldebyde-3-phosphate debydrogenase</td> <td>FOD817 STAALL</td> <td>gan</td> <td>SA011 2520</td> <td>SA046 2072</td> <td>X (core)</td> <td>17</td> <td>16</td> <td>g</td> <td>14</td>	Clyceraldebyde-3-phosphate debydrogenase	FOD817 STAALL	gan	SA011 2520	SA046 2072	X (core)	17	16	g	14
Interproduction EncloseFOD SIQ _ STAAU enceencloseSA011_222 SA04_2017SA046_2017 X (torle)X (torle) 22241819Lipid interprotein (3R)-hydroxymyristor)-[axy]- carrier-protein (dhydrataseFOD SM2_STAAU FOD SM2_STAAUfabZSA011_2232 SA046_0963SA046_015 X (core)22241819Acctoin reductase (SR)-hydroxymyristor)-[axy]- carrier-protein (dhydrataseFOD 3R9_STAAUSA011_0787 SA046_0963SA046_0105 X (core)10066Translation Constraint 500 strand 500 strand 500 strand 	Triosenboshate isomerase	FODE58 STAAL	tni A	SA011_2520	SA046_2072	X (core)	11	10	6	Q
Indice Tools 1, 2014 Gine Direction Gine Direction Function Function <t< td=""><td>Fnolse</td><td>FODSI1 STAAL</td><td>eno</td><td>SAO11_2522</td><td>SA046_2074</td><td>X (core)</td><td>22</td><td>74</td><td>18</td><td>19</td></t<>	Fnolse	FODSI1 STAAL	eno	SAO11_2522	SA046_2074	X (core)	22	74	18	19
Case FUD 6M2_STAAU fabZ SA011_1853 SA046_1611 X (core) 2 carrie-protein] dehydratase Acctoin reductase FOD3K9_STAAU SA011_0787 SA046_0963 X (core) 10 10 6 6 Carction reductase FOD1M7_STAAU ts SA011_0787 SA046_0015 X (core) 14 10 7 5 Elongation factor F FOD1M7_STAAU rps SA011_0284 SA046_1013 X (core) 14 10 7 5 Elongation factor P FOD246_STAAU rpt SA011_022 SA046_0849 X (core) 3 4 Elongation factor G FOD596_STAAU rpt SA011_1375 SA046_0987 X (core) 3 4 S0S ribosomal protein I3 FOD625_STAAU rpt SA011_1375 SA046_0987 X (core) 19 14 12 16 S0S ribosomal protein I25 FOD595_STAAU rpt SA011_1370 SA046_0987 X (core) 19 14 12 16 S0S	Linid metabolism	100001_011010	cho	5/1011_2524	5/1040_2070	A (core)	22	27	10	15
Chr. Instruction (abc) (fuct) FOD 3K9_STAAU ShOT [1005] ShOT [1005] X (core) 10 6 6 Accetoin reductase FOD 3K9_STAAU SA 011_0787 SA 046_0963 X (core) 10 6 6 Translation Elongation factor Ts FOD 1MT_STAAU tsf SA 011_0738 SA 046_0015 X (core) 21 20 9 16 Biongation factor Ts FOD 1MT_STAAU tsf SA 011_0732 SA 046_0045 X (core) 21 20 9 16 Biongation factor Ts FOD 1MT_STAAU tsf SA 011_0722 SA 046_0493 X (core) 3 4 7 Storman Interval FOD 595_STAAU tsf SA 011_1375 SA 046_0987 X (core) 13 16 20 Storman Interval FOD 595_STAAU tuff SA 011_1376 SA 046_0987 X (core) 19 14 12 16 20 Storman Interval FOD 595_STAAU tpl SA 011_1376 SA 046_0987 X (core) 19 14 12 16 20 16 20 20 24 <	(3P)-bydroxymyristoyl-[acy]-	FOD6M2 STAAL	fah7	SAO11 1853	SA046 1611	X (core)	2			
Actering Productase FDD3K9_STAAU SA011_0787 SA046_0963 X (core) 10 6 6 Translation Core 21 20 9 16 305 Ibosomal protein S4 FDD2F7_STAAU trsf SA011_038 SA046_0105 X (core) 21 20 9 16 305 Ibosomal protein S4 FDD2F7_STAAU trsf SA011_0384 SA046_0105 X (core) 21 20 9 16 305 Ibosomal protein S4 FDD28F_STAAU trsf SA011_0722 SA046_0888 X (core) 34 7 2 5 7 Elongation factor C FDD595_STAAU full SA011_1375 SA046_0898 X (core) 28 24 15 31 305 ribosomal protein S1 FDD595_STAAU full SA011_1375 SA046_0983 X (core) 19 14 12 16 20 305 ribosomal protein S1 FDD59_STAAU rpl SA011_1375 SA046_0993 27 40 5	carrier-protein] debydratase	1000002_011010	IdDL	5/1011_1055	5/1040_1011	A (core)	2			
Action Translation Short_GUOSD A (core) 10 10 10 0 0 Elongation factor Ts F0D1M7_STAAU tsf SAO46_0015 X (core) 21 20 9 16 305 ribosomal protein 54 F0D2F7_STAAU rpsD SAO11_0348 SAO46_1103 X (core) 3 4		FOD3KO STAALI		\$4011 0787	\$4046.0963	X (core)	10	10	6	6
Transcription FOD IM7_STAAU tsf SA011_0103 SA046_0015 X (core) 21 20 9 16 305 ribosomal protein 54 FOD2F7_STAAU rpsD SA011_0234 SA046_1103 X (core) 14 10 7 5 Elongation factor P FOD3E4_STAAU rpb SA011_0722 SA046_6494 X (core) 3 4 505 ribosomal protein 13 FOD4H6_STAAU rplC SA011_1276 SA046_0987 X (core) 7 2 5 7 Elongation factor G FOD596_STAAU rplC SA011_1376 SA046_0987 X (core) 19 14 12 16 305 ribosomal protein 125 FOD592_STAAU rplY SA011_1656 SA046_0987 X (core) 19 14 12 16 305 ribosomal protein 125 FOD592_STAAU rplY SA011_0100 SA046_093 27 40 5 24 DNA-directed RNA polymerase subunit beta FOD592_STAAU rpo8 SA011_1370 SA046_0932 27 40	Translation	100513_517010		5/1011_0787	3/1040_0303	X (colc)	10	10	0	0
Longaton lattor isInditing and the problem (attor)Inditing and the problem (attor)Anote (attor)Inditing and (attor) <thinditing (attor)<="" and="" th="">Inditing and</thinditing>	Flongation factor Ts		tef	\$4011 0103	\$4046 0015	X (core)	21	20	9	16
biologinal protein S4 fib/sbinlag/set	30S ribosomal protein S4		rncD	SA011_0103	SA046_0013	X (core)	14	10	7	5
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Flongation factor P		afn	SAO11_0722	SA046_0849	X (core)	3	10	1	5
biologinal protein DS 100-Fh0_SITMU0 ipc 5AO1_1200 5AO46_1936 X (core) 1 2 3 7 Elongation factor Tu FOD59C_STAAU trus SAO11_1375 SAO46_0988 X (core) 19 15 16 20 30S ribosomal protein S1 FOD625_STAAU rp SAO11_1375 SAO46_0987 X (core) 19 14 12 16 30S ribosomal protein S1 FOD625_STAAU rp SAO11_2426 SAO46_0987 X (core) 5 6 5 4 Transcription T Tomscriptional pleiotropic repressor CodY FOD1M4_STAAU rpoB SAO11_2426 SAO46_0912 12 11 4 7 DNA-directed RNA polymerase subunit beta FOD592_STAAU rpoB SAO11_1370 SAO46_0992 41 53 1 47 Replication and repair T FOD590_STAAU rpoC SAO11_2423 SAO46_0743 X (core) 7 14 1 6 Cell wall/membrane/envelop biogenesis T T 7 7 7 7 7 7 7 7<	50S ribosomal protein 13	FOD/H6 STAAU	rnlC	SA011_0722	SA040_0849	X(core)	7	4	5	7
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Flongation factor C		fue	SA011_1200	SA046_0988	X (core)	28	2	15	31
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Elongation factor Tu	FOD507 STAALI	tuf	SA011_1376	SA046_0987	X (core)	10	15	15	20
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	205 ribocomal protoin S1	EODG25 STAALL	rn	SA011_1570	SA046_0367	X (core)	10	14	10	16
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	505 ribosomal protein 125		rplV	SA011_1030	SA040_1850	X (core)	19	6	5	10
Trainer priorGTP-sensing transcriptional pleiotropic repressor CodYF0D1M4_STAAUcodYSA011_0100SA046_0012121147DNA-directed RNA polymerase subunit betaF0D591_STAAUrpoBSA011_1370SA046_099327400524DNA-directed RNA polymerase subunit β'F0D592_STAAUrpoCSA011_1371SA046_09924153147Replication and repairTTSA046_0743X (core)71416DNA polymerase III subunit βF0D5D0_STAAUdnaNSA011_2423SA046_0743X (core)71416Cell wall/membrane/envelop biogenesisF0D890_STAAUspoVGSA011_2423SA046_201722777Putative septation protein spoVGF0D890_STAAUspoVGSA011_0300SA046_0263777	Transcription	100033_31740	IpH	3A011_2420	3A040_2014	X (COLE)	5	0	5	4
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	CTP-sensing transcriptional pleiotropic repressor CodV		codV	\$4011 0100	\$4046 0012		12	11	4	7
DNA-directed RNA polymerase subunit β' FOD592_STAAU rpoC SA011_1371 SA046_0992 41 53 1 47 Replication and repair DNA polymerase subunit β FOD500_STAAU dnaN SA011_1409 SA046_0992 41 53 1 46 Cell wall/membrane/envelop biogenesis Putative septation protein spoVG FOD890_STAAU spoVG SA011_2423 SA046_0743 X (core) 7 14 1 6 Cell wall/membrane/envelop biogenesis Putative septation protein spoVG FOD890_STAAU tig SA011_0200 SA046_0263 7 7 7 Trigger factor FOD2K1_STAAU tig SA011_0428 SA046_0263 7 7 7 Trigger factor FOD2K1_STAAU tig SA011_0428 SA046_1149 X (core) 14 16 12 15 ATP-dependent proteinase chain FOD379_STAAU tig SA011_0657 SA046_1215 4 14 4 18 Urease accessory protein ureE FODAE9_STAAU groEL SA011_1310 SA046_1078 19 18 1 3 Endopeptidase FOD573_STAAU clpC SA011_1352 SA046_1011 17 23 Alkyl hydroperoxide reductase subunit C FODE08_STAAU ahpC SA011_1889 SA046_148 X (core) 2 5 4 2 Alkyl hydroperoxide reductase subunit C FODE08_STAAU ahpC SA011_1889 SA046_1458 X (core) 2 5 4 20	DNA directed DNA polymorase subupit beta	E0D501 STAAL	rpoP	SA011_0100	SA046_0002		27	10	-	24
DNA-chrected NAA polymetase submit βF0D592_5TAAUFpDcSA041_1571SA040_09924153147Replication and repairDNA polymerase III subunit βF0D5D0_STAAUdnaNSA011_1409SA046_0743X (core)71416Cell wall/membrane/envelop biogenesisPutative septation protein spoVGF0D890_STAAUspoVGSA011_2423SA046_20172Post-translational modification, protein turnover, chaperone functionsF0DA01_STAAUclpBSA011_0300SA046_202377Putative ATP-dependent protease proteinF0DA01_STAAUclpBSA011_0428SA046_1149X (core)14161215Putative ATP-dependent proteinase chainF0D379_STAAUclpLSA011_0657SA046_12154144418Urease accessory protein ureEF0DAE9_STAAUgroELSA011_1165SA046_107819181360 kDa chaperoninF0D531_STAAUgroELSA011_1310SA046_1078191813EndopeptidaseF0D573_STAAUclpCSA011_1352SA046_10111723413Alkyl hydroperoxide reductase subunit CF0D608_STAAUahpCSA011_1889SA046_1458X (core)2542Outer and the protein set of the protein	DNA-directed RNA polymerase subunit Beta		rpoC	SAO11_1370	SA040_0993		27 41	40 52	1	47
Replication and repartDNA polymerase III subuni βF0D5D0_STAAUdnaNSA011_1409SA046_0743X (core)71416Cell wall/membrane/envelop biogenesisPutative septation protein spoVGF0D890_STAAUspoVGSA011_2423SA046_20172Post-translational modification, protein turnover, chaperone functionsPutative ATP-dependent protease proteinF0DA01_STAAUclpBSA011_0300SA046_026377Trigger factorF0D2K1_STAAUtigSA011_0428SA046_1149X (core)14161215ATP-dependent proteinase chainF0D379_STAAUclpLSA011_0657SA046_1215414418Urease accessory protein ureEF0D4E9_STAAUgroELSA011_1165SA046_041133EndopeptidaseF0D573_STAAUclpCSA011_1310SA046_1078191813EndopeptidaseF0D573_STAAUclpCSA011_1352SA046_1011172342Alkyl hydroperoxide reductase subunit CF0D68_STAAUahpCSA011_1889SA046_1458X (core)2542Alkyl hydroperoxide reductase subunit CF0D68_STAAUahpCSA011_1889SA046_1458X (core)2542Alkyl hydroperoxide reductase subunit CF0D68_STAAUahpCSA011_1889SA046_1458X (core)2542	Poplication and repair	10D392_31AAU	TPOC	34011_1371	3A040_0352		41		1	47
Display programFrom Product of ConstraintsFrom Product of ConstraintsSAO40_0743X (cole)71410Cell wall/membrane/envelop biogenesisPutative septation protein spoVGF0D890_STAAUspoVGSAO11_2423SAO46_20172Post-translational modification, protein turnover, chaperone functionsPutative ATP-dependent protease proteinF0DA01_STAAUclpBSAO11_0300SAO46_0263777Trigger factorF0DA01_STAAUtigSAO11_0428SAO46_1149X (core)14161215ATP-dependent proteinase chainF0D379_STAAUtigSAO11_0657SAO46_1215414418Urease accessory protein ureEF0DAE9_STAAUureESAO11_1165SAO46_107819181360 kDa chaperoninF0D573_STAAUclpCSAO11_1310SAO46_1078191813EndopeptidaseF0D573_STAAUclpCSAO11_1352SAO46_1011172323Alkyl hydroperoxide reductase subunit CF0D608_STAAUahpCSAO11_1889SAO46_1458X (core)2542Alkyl hydroperoxide reductase subunit CF0DD608_STAAUahpCSAO11_1889SAO46_1458X (core)2542Alkyl hydroperoxide reductase subunit CF0D608_STAAUahpCSAO11_1800SAO46_1458X (core)2542	DNA polymorace III subunit β		dnaN	\$4011 1400	54046 0742	V (coro)	7	14	1	6
Putative septation protein spoVG F0D890_STAAU spoVG SA011_2423 SA046_2017 2 Post-translational modification, protein turnover, chaperone functions Putative ATP-dependent protease protein F0DA01_STAAU clpB SA011_0300 SA046_0263 7 7 7 Trigger factor F0D2K1_STAAU tig SA011_0428 SA046_1149 X (core) 14 16 12 15 ATP-dependent proteinase chain F0D379_STAAU clpL SA011_0657 SA046_1215 4 14 4 18 Urease accessory protein ureE F0DAE9_STAAU ureE SA011_1165 SA046_0411 3 60 kDa chaperonin F0D531_STAAU groEL SA011_1310 SA046_1078 19 18 1 3 Endopeptidase F0D573_STAAU clpC SA011_1352 SA046_1011 17 23 Alkyl hydroperoxide reductase subunit C F0D6Q8_STAAU ahpC SA011_1889 SA046_1458 X (core) 2 5 4 2	Call wall/membrane/envelop biogenesis	100000_31740	ullain	3A011_1409	37040_0743	X (COLE)	/	14	1	0
Platative septation protein sporeForbago STAACsporesporeSAO41_2423SAO42_20172Post-translational modification, protein turnover, chaperone functionsPutative ATP-dependent protease proteinFODA01_STAAUclpBSAO11_0300SAO46_026377Trigger factorFOD2X1_STAAUtigSAO11_0428SAO46_1149X (core)14161215ATP-dependent proteinase chainFOD379_STAAUclpLSAO11_0657SAO46_1215414418Urease accessory protein ureEFODAE9_STAAUureESAO11_1165SAO46_04113360 kDa chaperoninFOD531_STAAUgroELSAO11_1310SAO46_1078191813EndopeptidaseFOD573_STAAUclpCSAO11_1352SAO46_1011172342Alkyl hydroperoxide reductase subunit CFODE08_STAAUahpCSAO11_1889SAO46_1458X (core)2542Alkyl hydroperoxide reductase subunit CFODE08_STAAUahpCSAO11_1800SAO46_1458X (core)2542	Dutative contation protein cpoVC	FODOO STAALL	cnoVC	CA011 2422	SA046 2017			2		
Putative ATP-dependent protease protein to those, thaperone functions Putative ATP-dependent protease protein to FODA01_STAAU tig SA011_0300 SA046_0263 7 7 Trigger factor FODZN1_STAAU tig SA011_0428 SA046_1149 X (core) 14 16 12 15 ATP-dependent proteinase chain FOD379_STAAU clpL SA011_0657 SA046_1215 4 14 4 18 Urease accessory protein ureE FODAE9_STAAU ureE SA011_1165 SA046_0411 3 60 kDa chaperonin FOD531_STAAU groEL SA011_1310 SA046_1078 19 18 1 3 Endopeptidase FOD573_STAAU clpC SA011_1322 SA046_1011 17 23 Alkyl hydroperoxide reductase subunit C FODE08_STAAU ahpC SA011_1889 SA046_1458 X (core) 2 5 4 2 Alkyl hydroperoxide reductase subunit C FODE08_STAAU ahpC SA011_1890 SA046_1456 X (core) 2 5 4 2	Putative septation protein spove	robo90_STAAU	spove	SAUT1_2425	SAU40_2017			Z		
Trigger factorF0D2K1_STAAUtipSAO1_0428SAO4_020377Trigger factorF0D2K1_STAAUtigSAO1_0428SAO4_0128SAO4_012814161215ATP-dependent proteinase chainF0D379_STAAUclpLSAO1_0657SAO46_0115414418Urease accessory protein ureEF0DAE9_STAAUureESAO1_1165SAO46_04113360 kDa chaperoninF0D531_STAAUgroELSAO1_1310SAO46_1078191813EndopeptidaseF0D573_STAAUclpCSAO11_1352SAO46_10111723442Alkyl hydroperoxide reductase submit CF0D608_STAAUahpCSAO11_1889SAO46_1458X (core)2542Alkyl hydroperoxide reductase submit CF0DF08_STAAUahpCSAO11_1890SAO46_1458X (core)2542	Putative ATD dependent protocol protoin		clpP	\$4011 0200	51046 0262		7	7		
Insper lactor Iop Xn_STAL ig Sh01_042 Sh04_1149 X (cite) 14 16 12 15 ATP-dependent proteinase chain F0D379_STAAU clpL SA01_0657 SA046_1215 4 14 4 18 Urease accessory protein ureE F0DAE9_STAAU ureE SA01_1165 SA046_0411 3 3 60 kDa chaperonin F0D531_STAAU groEL SA01_1310 SA046_1078 19 18 1 3 Endopeptidase F0D573_STAAU clpC SA01_1352 SA046_1011 17 23 3 Alkyl hydroperoxide reductase submit C F0D68_STAAU ahpC SA01_11890 SA046_1458 X (core) 2 5 4 2	Trigger factor	FOD 2K1 STAND	tia	SACI1_0300	SACIA 11/0	X (coro)	14	16	12	15
Altr-dependent proteinase chainFOD579_31AAUClpLSAO1_0057SAO46_1215414418Urease accessory protein ureEFODAE9_STAAUureESAO1_1165SAO46_04113360 kDa chaperoninFOD531_STAAUgroELSAO1_1310SAO46_1078191813EndopeptidaseFOD573_STAAUclpCSAO1_1352SAO46_101117233Alkyl hydroperoxide reductase subunit CFOD68_STAAUahpCSAO1_1889SAO46_1458X (core)2542	ATD dependent proteinase chain	EOD2RI_SIAAU	clpl	SA011_0420	SA040_1149	A (LOIE)	14	10	12	10
FODDAES_STAAU unee SAO11_1165 SAO46_0411 3 60 kDa chaperonin FOD531_STAAU groEL SAO11_1310 SAO46_1078 19 18 1 3 60 kDa chaperonin FOD531_STAAU groEL SAO11_1310 SAO46_1078 19 18 1 3 Endopeptidase FOD573_STAAU clpC SAO11_1352 SAO46_1011 17 23 Alkyl hydroperoxide reductase subunit C FOD6Q8_STAAU ahpC SAO11_1889 SAO46_1458 X (core) 2 5 4 2		FODAEO STAAU	CIPL	SAUTI_0057	SA040_1215		4	14	4	10
of KDa ChaperformF0D531_51AAUgroetSAO11_1310SAO46_1078191813EndopeptidaseF0D573_STAAUclpCSAO11_1352SAO46_10111723Alkyl hydroperoxide reductase subunit CF0D6Q8_STAAUahpCSAO11_1889SAO46_1458X (core)2542	Go kDa chaperonin	FODE21 STAAU	ureE	SAUTI_1105	SA040_0411		10	Э 10	1	2
Endopeptidase F0D5/3_51AAU Cipc SA011_1522 SA046_1011 17 23 Alkyl hydrogrogydd reductase subunit C F0D6Q8_STAAU ahpC SA011_1889 SA046_1458 X (core) 2 5 4 2	ou kua chaperonni Endepentidase	FUD331_STAAU	groel	SAUTI_1310	SAU40_1078		19	10	1	٢
$\frac{1}{1000} = \frac{1}{1000} = 1$	Eliuopepuidase Allail hydroporovido roductore cubunit C	FUDS/3_SIAAU	abpC	SAUTI_1352	SAU40_1011	V (coro)	1/	23	4	2
	Allad budroperovide reductase subunit C	FODDE2 STAAU	anpe	SAUTI_1009	SA040_1458	X(011)	2	5	4	2

Table 1 (Continued)

Description ^a	Accession ^b	Gene name ^c	011 ^d	O46 ^e	SERPA ^f	No. of peptides	No. of peptides O46 curd ^h	No. of peptides	No. of peptides
						UTI Cuita"		UTT IIIIK	040 111116
Thioredoxin reductase	F0D8B3_STAAU	trxB	SAO11_2446	SAO46_2742	X (011)	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	
Inorganic ion transport and metabolism									
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	mqo	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
Signal transduction									
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		
Virulence-defense mechanism									
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			
Unknown function									
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		

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

^b Accession number on Uniprot.

^c Gene name.

^d Coding sequence numbers corresponding to the identified proteins in *S. aureus* O11.

^e Coding sequence numbers corresponding to the identified proteins in *S. aureus* O46.

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

^g Number of peptides identified in sample from curd isolated after infection by 011.

^h Number of peptides identified in sample from curd isolated after infection by O46.

ⁱ Number of peptides identified in sample from O11 grown *in vitro* in ewe milk.

^j 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 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 strainspecific 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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