Chapter 2

Efficient production of secreted staphylococcal antigens in a non-lysing and proteolytically reduced *Lactococcus lactis* strain

Jolanda Neef, Danny G.A.M. Koedijk, Tjibbe Bosma, Jan Maarten van Dijl, Girbe Buist

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Abstract

Cell surface-exposed and secreted proteins are attractive targets for vaccination against pathogenic Gram-positive bacteria. To obtain sufficient amounts of such antigens, efficient protein production platforms are needed. In this study a pipeline for the production and purification of surface-exposed and secreted antigens of the Gram-positive bacterial pathogen *Staphylococcus aureus* is presented. Cytoplasmic or extracellular production of *S. aureus* antigens was achieved using the *Lactococcus lactis* strain PA1001, which lacks the major extracellular protease HtrA and the autolysin AcmA to minimize proteolysis and cell lysis, respectively. For most tested *S. aureus* antigens, secretory production directed by the signal peptide of the major secreted protein Usp45 of *L. lactis* resulted in higher yields than intracellular production without a signal peptide. Additionally, secretory production of His-tagged antigens allowed their facile one-step purification from the growth medium by metal affinity chromatography. For three of the purified antigens biological activity was confirmed through enzyme activity assays. We furthermore show that the present pipeline can be used to produce staphylococcal antigens with an N-terminal AVI-tag for site-specific labeling with biotin, or a C-terminal cell wall-binding domain for cell surface display. We conclude that our *L. lactis*-based pipeline allows the efficient production of *S. aureus* antigens and their subsequent purification in one step.
Introduction

For the production of vaccines against Gram-positive bacterial pathogens, the application of surface-exposed proteins has been shown to be very efficient (1). For the pathogen *Staphylococcus aureus* many studies to develop protective vaccines or antibodies based on the recognition of a surface protein have been undertaken (2). Using different proteomics approaches potentially invariant target proteins have been identified. These are either surface-exposed, secreted or both (3-5). Immuno-proteomics approaches have been applied to determine variations in the specific immune responses to these proteins in healthy and infected humans (6). This information pinpoints potential targets for immunotherapy against resistant *S. aureus* strains that now need to be validated through Enzyme-Linked Immuno Sorbent Assay, Luminex Flow Cytometry (7,8) protein arrays (9) or electrical protein array chips (10). All these techniques have in common that potential target proteins need to be overproduced and purified, which underscores the need for suitable expression systems.

In most previous studies, protein targets have been (over-)produced in *E. coli* using the IPTG-inducible T7 promoter (9). Overexpression of heterologous proteins in *E. coli* can be very efficient, but problems such as low yields, the formation of inclusion bodies and protein degradation are often encountered (11,12). To avoid such problems, different solutions have been proposed, including the use of specific growth regimen to increase expression, the solubilization of inclusion bodies with urea or other denaturing compounds, and the use of specific strains with reduced proteolytic activity. Furthermore, *E. coli* produces lipopolysaccharide (LPS), generally referred as endotoxin, which needs to be removed before produced proteins can be used in clinical applications (12).

Besides *E. coli*, several Gram-positive bacteria have been used for heterologous protein production, such as various *Bacillus* species (11,12) and *Lactococcus lactis*. The latter bacterium offers several advantages. *L. lactis* is a facultative anaerobe that is easy to culture. Importantly, compared to *Bacillus* species, *L. lactis* is barely proteolytic as it produces only one major extracellular protease, designated HtrA (13), and it secretes only one abundant protein known as Usp45 (unidentified secreted protein of ~45 kDa) (14). Furthermore,
heterologous proteins can be secreted from *L. lactis* in a folded and active state (15,16), which facilitates easy downstream processing. Lastly, the generally-regarded as safe status, the availability of the complete genome sequence and a wealth of genetic tools for cloning and strain optimization make *L. lactis* ideally suited for the expression of heterologous proteins (17,18). This is exemplified by the efficient production and secretion of His-tagged proteins from *Listeria monocytogenes* in *L. lactis* (19).

Different *L. lactis* expression systems have been developed of which the Nisin-Controlled Expression (NICE) system has been shown to be most efficient (20,21). In this system, the lantibiotic Nisin is used as an inducer, which is sensed by the NisK histidine protein kinase located in the cell envelope (Fig. 1). NisK subsequently phosphorylates the response regulator NisR, which subsequently activates the transcription of (heterologous) genes that have been cloned downstream of the *nisA* promoter (14,21). Various vectors have been produced for use in the NICE system, some of which include the signal sequence of the secreted Usp45 protein (14,22).

Figure 1. Schematic representation of nisin-induced expression and secretion of tagged *S. aureus* antigens by *L. lactis* PA1001. NisK is the sensor protein that phosphorylates (Pi) the response regulator NisR and activates the expression of the target gene. The expressed antigen is C-terminally fused to a His-tag (H) and can be N-terminally fused to an AVI-tag for enzymatic biotinylation. The antigen will be secreted into the growth medium via the general secretion (Sec) pathway when fused to the signal peptide (SP) of the Usp45 protein of *L. lactis*. Instead of the His-tag a protein anchor (PA) can be fused to the antigen for cell surface display.

The proteolytic degradation of heterologous proteins can be a problem for stable protein production in *L. lactis* (22), but this can be overcome by deletion of the *htrA* gene (23). Another potential problem is autolysis of protein-producing cells, which leads to the release of cytoplasmic proteins into the
medium and poses a challenge for the purification of secreted target proteins. Autolysis in *L. lactis* is mainly caused by the peptidoglycan hydrolase AcmA (24). Accordingly, deletion of the acmA gene was shown to result in a complete loss of autolysis even after 7 days of culturing (25).

The aim of this study was to develop a pipeline for the expression and purification of tagged surface-exposed and secreted proteins from *S. aureus*. For this purpose, we used the *S. aureus* proteins Aly (SA2437), LytM (SA0265), Nuc (SA0746), SA0620, SA2097, and IsaA (SA2356) as reporters. These staphylococcal proteins are potentially relevant antigens, because in a previous study it was shown that they are invariably present in the exoproteomes of 25 different clinical *S. aureus* isolates (3). Specifically, we compared the efficiencies of cytoplasmic and secretory production of these reporter proteins in *acmA htrA* double mutant *L. lactis* cells. In brief, the results show that such double mutant cells allow the efficient secretion of *S. aureus* proteins.

**Materials and Methods**

**Bacterial strains and growth conditions**

Strains and plasmids are listed in Table 1. *L. lactis* strains were grown at 30ºC in M17 broth (Oxoid Limited, Hampshire, United Kingdom) with 0.5% glucose (w/v) (GM17). When necessary, the medium was supplemented with chloramphenicol (5 μg/ml). The *S. aureus* strains N315 and Newman were grown overnight at 37ºC as standing cultures using Tryptone Soy Broth (TSB, Oxoid Limited).

**General molecular biology**

Enzymes and buffers were from New England Biolabs (Ipswich, United Kingdom) and Fermentas (Landsmeer, The Netherlands). For cloning experiments T4 DNA ligase was used. Genomic DNA of *S. aureus* N315 was isolated with the Genelute bacterial genomic DNA kit (Sigma-Aldrich, Zwijndrecht, The Netherlands) according the manufacturer’s protocol with the following modifications. *S. aureus* cells were lysed using Lysostaphin (5 U/μl, Ambi Products, Lawrence, USA) in Gram-Positive Lysis Buffer for 20 min at 37 ºC, followed by addition of the lysis solution both provided with the kit.
PCR reactions were performed with a DNA Engine Tetrad (MJ Research Inc., St. Bruno, Canada) or a Bio-rad C1000 Thermal cycler (Richmond, CA). Primers used in this study were obtained from Eurogentec (Maastricht, The Netherlands) (Table 2). The polymerases Taq (Life Technologies, Bleiswijk, The Netherlands), PWO (Roche, Woerden, The Netherlands), Phusion Hot Start (Thermo Fisher Scientific, Wilmington, Delaware USA) were used according to the manufacturer’s protocols. PCR products were purified using the High Pure PCR product purification kit from Roche. *L. lactis* cells were lysed with Lysozyme (4 mg/ml, Sigma-Aldrich) in kit solution A, 10 min at 55ºC, followed
by addition of the lysis buffer provided with the Invisorb Spin Plasmid Mini Two kit (Invitex GmbH, Berlin, Germany). Nucleotide sequence analyses were performed by Eurofins DNA (Ebersberg, Germany). Electrottransformation of *L. lactis* was performed using a Gene pulser (Bio-Rad Laboratories) as described by (26).

Table 2: Primers used in this study

<table>
<thead>
<tr>
<th>Primen</th>
<th>5’→3’ nucleotide sequence</th>
<th>Restriction enzyme</th>
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<tr>
<td>His_PA.fw</td>
<td>CATCACCATCACCATCACAGAGACCTTACCAGAAGACAAGGAAATAC</td>
<td></td>
</tr>
<tr>
<td>P_His_PA.rev</td>
<td>GCCAGCTAAGACACCTGACACG</td>
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| 2097.fw | CCCGGCTCTCCATGGGAGCTGAAAATTATACAAATTAC | BsmBI
| 0620.fw | CCCGTCTCCATGGGATCTACACAAACATACTACAAAT | BsmBI
| pPA180.rev | GCCTGGTTTTTCAATTGGTTTCAAAG | |
| Avi-tag.fw | CATGAGGTGTTAAGAGATATTGCTGAGGTCAGAATATCGAATGAC | |
| Avi-tag.rev | CATGATTTGTCGTTTTGACAGCTGAGGATAATCTGTTTAAACC | |
| AVIIIsA.fw | CCCGGTCTCCATGAGATGGGTAAAACAGATATTTCCGAGGC | BsmBI
| AVIIIsA.rev | CGGCCGCAGCTTGTATGATGCTT | HindI
| Aly224.fw | CGTACAGTCTCATGACGACACCTCAAAAAGATACTACAG | BsaI
| Aly224.rev | CCAGAAGACGTTTCTTTACCTCTGTAATTATAGAAAATCTCAG | BbsI
| 0620224.fw | CTGACAGCTCTCATGACACTCAACAAACATACGATCAATTCTCAG | BsaI
| 0620224.rev | CCAGAAGACGTTTCCATGGATGACAGCTGAGAACATACAAATTTCTG | BbsI
| 2097224.fw | CTGACAGGTCTCTCATGACGCTGAAAAATATTACAAATTACCAAC | BsaI
| 2097224.rev | CCAGAAGACGTTTCCATGACACCTCAAAAAGATACTACAG | BbsI
| Nuc224.fw | CTGACAGGTCTCTCATACACACAAACAGATAACGCTGAATAG | BsaI
| Nuc224.rev | CCAGAAGACGTTTCTTTACCTGACGTTAATCTCTCC | BbsI
| LytM224.fw | CTGACAGGTCTCTCATGACGCAAGAAAAACACCCAAACACAC | BsaI
| LytM224.rev | CCAGAAGACGTTTCTCTTCTACACCTCCCAAGTATGACGCTTGGGTACTAC | BbsI
| AlyHis.fw | CACTCAGGCTCTCCATGGGAGATAACCTCAAAAAGATACTAC | BsaI

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Construction of expression plasmids

For construction of pPA224::his primer His_PA.fw was used in combination with P_His_PA.rev, using pPA224 as template. The resulting PCR product was ligated and transferred to L. lactis PA1001.

For extracellular production of Aly, LytM, Nuc, SA0620 and SA2097 fused to the PA cell wall anchor (i.e. three tandem LysM repeats of L. lactis AcmA), the respective genes where amplified using primer combinations Aly224.fw/Aly224.rev, LytM224.fw/LytM224.rev, Nuc224.fw/Nuc224.rev, SA0620224.fw/SA0620224.rev and SA2097224.fw/SA2097224.rev. The resulting PCR products and receiving plasmid pPA224 were cut with the restriction enzymes Bsai and BbsI. After ligation, the resulting vectors were used to transform L. lactis PA1001.

C-terminally His-tagged fusions for intracellular production of Aly, LytM, Nuc, SA0620 and SA2097 were PCR-amplified with primer combinations AlyHis.fw/AlyHis.rev, LytMHis.fw/LytMHis.rev, NucHis.fw/NucHis.rev, 0620His.fw/0620His.rev and 2097His.fw/2097His.rev using chromosomal DNA of S. aureus N315 as the template. Amplified DNA fragments were cleaved with Bsai resulting in Ncol and EcoRI overhangs. The receiving plasmid pPA180 was cleaved with Ncol and EcoRI. After ligation, the resulting plasmids were used to transform L. lactis PA1001.

A C-terminal His-tag fusion of Aly for extracellular production was constructed by cleaving pPA180::aly::his₆ with Ncol and HindIII and ligation into pNG400 cut
with the same enzymes, resulting in pNG400::aly::his₆. For construction of pNG400::lytM::his₆ the same strategy was used. The construction for extracellular production of Nuc-His₆ was made by digestion of pPA180::nuc::his₆ with XhoI and Ncol and ligation into pNG400 cut with the same enzymes. For construction of C-terminally His-tagged fusions of S. aureus SA2097 and SA0620, PCR reactions were performed on pPA180::sa2097::his₆ and pPA180::sa0620::his₆ using primers 2097.fw and 0620.fw, respectively in combination with pPA180.rev. The amplified DNA fragment was cut with HindIII and BsmBI and ligated into pNG400 cleaved with Ncol and HindIII. For N-terminal fusion of avi to isaA::his in pET24d::isaA::his₆, primers Avi-tag.fw and Avi-tag.rev were diluted in ligation buffer (Fermentas) and heated to 90ºC for 15 min. The temperature was decreased slowly to room temperature resulting in annealing of the primers generating Ncol overhangs. pET24::isaA::his₆ cut with Ncol was ligated with avi resulting in removal of the Ncol site, and the respective ligation mixture was used to transform E. coli BL21DE3. Subsequently the avi::isaA::his₆ fusion was cloned into pNG400 using primers AVIIasaA.fw and AVIIasaA.rev with pET24::avi::isaA::his₆ as a template. The resulting PCR product was cut with BsmBI and HindIII and ligated into pNG400 cut with Ncol and HindIII.

**Protein production, detection, isolation and quantification**

Cultures of L. lactis were diluted 1:20 in fresh GM17 medium. Induction of P<sub>nisa</sub> was performed at OD<sub>600</sub> ~0.5 for 16 hours by adding the culture supernatant (1:1000) from an overnight culture of the nisin-producing strain L. lactis NZ9700 as described (16). Cells and growth medium were separated by centrifugation. Proteins in the growth medium fraction were precipitated with 10% trichloroacetic acid (TCA; 10% w/v final concentration) and dissolved in LDS buffer (Lithium Dodecyl Sulfate, Life Technologies). Cells were resuspended in LDS buffer and disrupted by bead-beating with 0.1 um glass beads (Biospec Products, Bartlesville, USA) using a Precellys24 bead beater (Bertin Technologies, Montigny-le-Bretonneux, France). Protein samples were incubated at 95ºC for 10 min, separated by LDS-PAGE using precast 10% NuPage gels (Invitrogen) and stained with SimplyBlue™ SafeStain (Life Technologies). For Western Blotting analyses, proteins separated by LDS-PAGE were blotted onto a nitrocellulose membrane (Protran®, Schleicher & Schuell,
Dassel, Germany). Immunodetection was performed using anti His-tag antibodies (Life Technologies), rabbit polyclonal antibodies raised against PA (Eurogentec), or polyclonal antibodies against AVI (Genscript, Piscataway, USA). Bound antibodies were visualized using fluorescently labeled secondary antibodies (IRDye 800 CW from LiCor Biosciences, Nebraska, USA). Membranes were scanned for fluorescence at 800 nm using the Odyssey Infrared Imaging System (LiCor Biosciences).

For protein isolation cells were disrupted by bead-beating in binding buffer (20 mM NaPi, pH7.4, 0.5 M NaCl, 50 mM Immidazole). Extracellular production of proteins were precipitated with TCA. Cleared lysate or supernatant fractions were applied to Ni-Sepharose Fast Flow (GE Healthcare Life Sciences, Diegem, Belgium) and washed with 3 column volumes of binding buffer. His-tagged proteins were eluted with 3 column volumes of binding buffer containing 500 mM imidazole. Fractions containing the purified His-tagged proteins were pooled and dialyzed against PBS.

Protein concentrations were determined with the DC Protein Assay (BioRad) using Bovine Serum Albumin (Sigma Aldrich) as a standard or with the Nanodrop ND-1000 (Thermo Fisher Scientific) using calculated extinction coefficients (http://www.biomol.net/en/tools/proteinextinction.htm).

**Protein activity assays**

Peptidoglycan hydrolase activity was detected by a zymogram staining as described previously (25). Nuclease activity was tested by mixing ~50 ng of Nuc-His$_6$ or 200 ng DNAsase (Roche) with 25 μg of chromosomal DNA of *S. aureus* in buffer L (10 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM dithioerythritol, final concentration) for restriction endonucleases (Roche). Mixtures were incubated for 5 min at room temperature. Enzyme activity was stopped by adding DNA-loading buffer and DNA degradation was monitored using 1.0% agarose gels.

Peptidoglycan-degrading activity of LytM was determined by measuring the reduction in OD$_{600}$ after 90 min incubation of *S. aureus* Newman cells (diluted to an OD$_{600}$ of 0.5 in PBS) with 50 μg of purified LytM at 37°C. *S. aureus* cells used were grown in TSB and washed with and dissolved in PBS. As a control, the cells were incubated with PBS.
Biotinylation assay
For biotinylation of AVI-IsaA-His$_6$ produced in *L. lactis*, medium fractions of overnight induced cell cultures were 10-fold concentrated using TCA precipitation. As a negative control His$_6$-FtsL was used. The precipitated protein pellets were biotinylated at 30°C for 1 hour using BirA500 (Avidity, Aurora, USA) according to the manufacturer’s instructions. Biotinylation was analyzed using LDS-PAGE combined with Western Blotting. After blocking the nitrocellulose membrane in 5% BSA in TBS (50 mM Tris, 150 mM NaCl, pH 8), the biotinylated proteins were targeted with Streptavidin conjugated to Alkaline Phosphatase (AP; Sigma-Aldrich) in 1:5,000 dilution. Streptavidin binding was visualized using a 50x diluted NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyolphosphate p-toluidine salt) stock solution (Roche) in AP-buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl$_2$). As a control also the His-tag was detected as described above.

Results

Reduced lysis and protein degradation using an autolysis and proteolysis deficient strain of *L. lactis* as a production host.
As a first approach to determine whether an acmA htrA double mutation can be beneficial for the production of *S. aureus* antigens, the His-tagged fusion protein LytM-His$_6$ was used. This reporter protein was furthermore fused to the signal peptide of Usp45 for secretion into the growth medium by expression from pNG400. As shown in Figure 2, the LytM-His$_6$ fusion protein was effectively produced and secreted both by *L. lactis* NZ9000 (acmA- and htrA-proficient) and *L. lactis* PA1001 (acmA- and htrA-deficient). Notably, about 50% of the fusion protein was retained in the cell probably due to cell wall retention by the cell wall-binding of LytM (29). Western blotting analysis of the cell and growth medium fractions showed that the LytM-His$_6$ fusion protein is subject to proteolytic degradation in strain NZ9000 as bands with a higher electrophoretic mobility than that of the full-size fusion protein were detected (Fig. 2, lanes marked N). No degradation of the LytM-His$_6$ protein was observed in the cell and growth medium fractions of *L. lactis* PA1001 (Fig. 2, lanes marked P). Furthermore, comparison of the growth medium fractions of NZ9000 and PA1001 showed that fewer background bands were present in the
medium of strain PA1001, suggesting that this strain is less prone to autolysis (Fig. 2A). Taken together, these results show that the \textit{L. lactis} PA1001 strain is preferable over the NZ9000 strain for the secretory production of \textit{S. aureus} antigens, like LytM-His\textsubscript{6}. Therefore this strain was also selected for the expression of the other His-tagged \textit{S. aureus} proteins used in this study.

**Figure 2.** Reduced degradation of LytM-His\textsubscript{6} and loss of cell lysis upon expression in \textit{L. lactis PA1001}. Cell and growth medium fractions of \textit{L. lactis} NZ9000 (N) or \textit{L. lactis} PA1001 (P) producing LytM-His\textsubscript{6} were analyzed by LDS-PAGE. Gels were either stained with Simply blue safe stain (a) or used for Western blotting (b) with anti-Histidine antibodies. Grey arrow heads indicate mature LytM-His\textsubscript{6}, black arrow heads indicate LytM-His\textsubscript{6} dimers, white arrow heads indicate degradation products detectable after secretion by strain NZ9000. The molecular weight of marker proteins is indicated (in kDa).

**Efficient intracellular or secretory production of His-tagged staphylococcal antigens in \textit{L. lactis}.

To further explore the potential of \textit{L. lactis} PA1001 for the production of \textit{S. aureus} antigens, the genes for the Aly, LytM, Nuc, SA0620, SA2097 and IsaA proteins were cloned in the NICE system using plasmid pPA180 for intracellular production, or plasmid pNG400 for secretory production driven by the Usp45 signal peptide. Notably, for cloning in either vector, the authentic signal sequences of the different \textit{S. aureus} proteins were removed. Furthermore, the genes were cloned in such a way that the expressed \textit{S. aureus} antigens were provided with a C-terminal His-tag. As shown by LDS-PAGE and subsequent Simply Blue staining (Fig. 3A) or Western Blotting with antibodies against the
His-tag (Fig. 3B), the intracellularly produced His\textsubscript{6}-tagged fusion proteins (lanes marked ‘Cytoplasmic’) were exclusively detectable in the cell lysates. Only in case of the SA0620-His\textsubscript{6} fusion, a degradation product of \(~17\) kDa was detected. Furthermore, expression of SA2097-His\textsubscript{6} was found to be relatively low compared to the other \textit{S. aureus} antigens.

When the His-tagged \textit{S. aureus} proteins were fused to the signal peptide of Usp45, all His-tagged \textit{S. aureus} proteins were secreted into the growth medium except for SA0620-His\textsubscript{6} (Fig. 3, lanes marked ‘+ssUsp45’). In addition, substantial amounts of these proteins were detectable in the cell lysates. A similar degradation product of the SA0620-His\textsubscript{6} fusion protein as observed upon intracellular production was observed also upon secretory production.

Western analysis using anti-His antibodies showed that the full size SA0620-His\textsubscript{6} is present in the cell lysate but not in the supernatant. Relatively high levels of production and secretion were observed for LytM-His\textsubscript{6} and Nuc-His\textsubscript{6}, while relatively inefficient secretion was observed for Aly-His\textsubscript{6}. The SA2097-His\textsubscript{6} fusion protein was produced at relatively low levels but its secretion was efficient (Fig. 3). Interestingly, for all secreted fusion proteins we detected slowly migrating bands, which possibly represent multimeric forms (Fig. 3B).

Altogether, these results show that \textit{L. lactis} can be used for intracellular and secretory production of antigens that are naturally secreted by \textit{S. aureus}. Importantly, secretory production leads to higher yields and is therefore the preferred option to produce the investigated \textit{S. aureus} antigens.

**Efficient production and secretion of staphylococcal antigens tagged with a cell wall anchor in \textit{L. lactis}**

In a next series of experiments the Aly, LytM, Nuc, SA0620, SA2097 and IsaA proteins were cloned in the NICE system using pPA224. By doing so, these proteins were fused to the PA cell wall anchor sequence, which allows their cell surface display in \textit{L. lactis} (16,30). Upon nisin induction, clear production and secretion of Aly-PA, LytM-PA and Nuc-PA was observed upon LDS-PAGE and Simply Blue staining of the separated proteins (Fig. 3A). A somewhat lower production and secretion level was observed for SA2097-PA. In contrast, the full-size SA0620-PA fusion protein of 51 kDa remained undetected in the supernatant fraction.
Figure 3. Analysis of cytoplasmic and secreted production of His-tagged or PA-tagged \textit{S. aureus} antigens by \textit{L. lactis} PA1001. (a) Cells and growth medium fractions of \textit{L. lactis} PA1001 expressing cytoplasmic (-ssUsp45) or secreted (+ssUsp45) His-tagged or PA-tagged staphylococcal antigens were analyzed by LDS-PAGE. Gels were stained with Simply blue safe stain. An additional band of approximately 37 kDa is indicated by a star. Usp45 in the growth medium fraction is marked. Control, samples of an uninduced \textit{L. lactis} PA1001 strain. The molecular weight of marker proteins is indicated (in kDa). (b) Cells and growth medium fractions of \textit{L. lactis} PA1001 expressing His-tagged cytoplasmic (-ssUsp45) or secreted (+ssUsp45) staphylococcal antigens were analyzed by Western Blotting using anti-Histidine antibodies. Grey arrow heads indicate the produced fusion proteins, black arrow heads indicate dimers, white arrow heads indicate degradation products of the produced proteins.
Instead, a specific degradation product of around 37 kDa was observed upon LDS-PAGE. Western Blotting analysis using specific anti-PA antibodies showed the presence of multiple degradation products for this fusion protein (Fig. 4), indicating that it is prone to proteolysis even in the PA1001 strain. It should be noted that in all supernatant fractions the Usp45 protein (~50 kDa) and an additional band of approximately 37 kDa (marked with a star in Fig. 3A) were detectable. No signal was obtained for proteins of this size in the Western analysis using antibodies raised against the PA anchor (Fig. 4), indicating that the 37 kDa band does not contain the PA domain or fragments thereof.

Figure 4. Western analysis of expressed and secreted PA-fusions of staphylococcal proteins produced by *L. lactis* PA1001. Cell lysates and culture supernatant samples of *L. lactis* PA1001 expressing the PA-fusions shown in Figure 3 were analyzed by Western Blotting using anti-PA antibodies. Grey arrow heads indicate the produced fusion proteins. The molecular weights of marker proteins are indicated (in kDa).

Comparison of the cell lysates and the supernatant fractions of the strains producing the PA-fusions showed that nearly 50% of each produced PA-fusion
protein is located in the respective cell lysate (Fig. 4). This is most likely due to the presence of the PA-domain as a PA-His$_6$-tag fusion composed of only the PA anchor and a His-tag showed a similar distribution in the growth medium and cell fractions upon expression in the PA1001 strain (Fig. 5). Moreover, the cell-associated PA-His$_6$ fusion could be stripped from the cells by boiling the bacterial cell pellet in LDS loading buffer, which indicates that this fusion was associated to the cell wall. Although more proteins are released from the cells upon boiling in LDS loading buffer, the PA-His$_6$ fusion is the most abundantly released protein, indicating that its release is not due to cell lysis. Together, these observations indicate that the *S. aureus* proteins fused to the PA anchor are translocated across the cell membrane. Subsequently, about 50% of each produced PA-fusion protein is retained in the cell wall while the other 50% is released into the growth medium.

Figure 5. Secretion and cell wall binding of His$_6$-PA produced by *L. lactis* PA1001. LDS-PAGE analysis of His$_6$-PA produced by *L. lactis* PA1001 and subsequent protein detection with Simply Blue staining. Lane 1, TCA-precipitated supernatant of *L. lactis* PA1001 producing His$_6$-PA. Lanes 2 and 3, cell lysates of PA1001 producing His$_6$-PA before (lane 2) or after (lane 3) stripping the cells with LDS buffer. Lane 4, Proteins released from the cell wall upon stripping with LDS buffer.

**Secretion of soluble AVI-IsaA-His$_6$ by *L. lactis*.
To be able to label staphylococcal antigens at a specific site, for example with a fluorescent dye, the possibility to use an AVI-tag was explored by fusing this tag to the N-terminus of the IsaA-His$_6$ protein. The AVI-tag is a unique 15 amino acid peptide (GLNDIFEAQKIEWHE) to which biotin can be coupled by the biotin ligase (BirA) from *E. coli*. As shown by LDS-PAGE the AVI-IsaA-His$_6$ fusion
protein was efficiently secreted from *L. lactis* PA1001 (Fig. 6A). Furthermore, the secreted protein contained both the AVI-tag and the His-tag as verified by Western Blotting using specific antibodies (Fig. 6A). Next, the AVI-IsaA-His$_6$ protein produced by *L. lacis* was tested for biotinylation (Fig. 6B). To this end, cells and growth medium were separated by centrifugation, and the growth medium fraction was TCA precipitated. Next, the AVI-IsaA-His$_6$ protein was resuspended in biotinylation buffer containing biotin and incubated with BirA. Biotinylation was assayed using streptavidin conjugated with alkaline phosphatase.

**Figure 6. Extracellular production and biotinylation of AVI-IsaA-His$_6$ in *L. lactis* PA1001.**

(a) For the detection of extracellularly produced AVI-IsaA-His$_6$ by *L. lactis* PA1001, cells (C) and TCA-precipitated growth medium fractions (S) of non-induced (-) and induced (+) cell cultures were separated by LDS-PAGE. Subsequently, the gel was stained with Simply Blue (SB), or used for Western Blotting with Anti-Histidine or Anti-AVI antibodies. The molecular weight of marker proteins is indicated. (b) TCA precipitations of AVI-IsaA-His$_6$ (IsaA) and His$_6$-FtsL (FtsL) were incubated without (-) or with (+) BirA. Proteins were separated on an LDS-PAGE gel and blotted on nitrocellulose membranes. Biotinylation was analyzed by binding of Streptavidin-AP and visualized using NBT/BCIP. As a control Western Blotting was performed using anti-Histidine antibodies.
As shown by alkaline phosphatase activity assays, this procedure resulted in efficient detection of the biotinylated AVI-IsaA-His<sub>6</sub> (Fig. 6B). In contrast, the His-tagged control protein FtsL, which lacked the AVI-tag, was not detectable with the streptavidin-alkaline phosphatase conjugate. A background signal of approximately 25 kDa was detected in all TCA precipitated medium fractions, both in the BirA treated and untreated samples indicating that this signal is not obtained due to biotinylation of a naturally secreted lactococcal protein. Together, these findings show that even <i>S. aureus</i> proteins with multiple tags can be efficiently secreted using <i>L. lactis</i>.

Facile protein purification from cytoplasmic or supernatant fractions of <i>L. lactis</i>.  
To assess whether the intracellularly or secreted <i>S. aureus</i> antigens could be readily purified from overproducing <i>L. lactis</i> cells grown in 500 ml of GM17 broth, we performed metal affinity chromatography. As shown in Figure 7, the overproduced LytM-His<sub>6</sub> was successfully isolated from either disrupted cells or the growth medium.

**Figure 7. Purification of LytM-His<sub>6</sub> from <i>L. lactis</i> cells or the growth medium.**
Intracellularly produced LytM-His<sub>6</sub> protein was isolated from cells (C), and extracellularly produced LytM-His<sub>6</sub> from the growth medium (M) as detailed in the Materials and Methods. The flow through (F) fractions, wash and elution fractions were analyzed by LDS-PAGE. The position of the LytM-His<sub>6</sub> protein is indicated by the arrow.

Subsequent quantification of the amounts of purified protein showed that about 4-fold more LytM-His<sub>6</sub> was purified from the growth medium than from
the cells (Table 3). Similar amounts of Nuc-His\textsubscript{6} were obtained from the growth medium of overproducing cells, but in this case the amount of protein purified from the cells was about 1.5-fold higher than observed for LytM-His\textsubscript{6}. Compared to LytM-His\textsubscript{6} and Nuc-His\textsubscript{6}, significantly lower amounts of Aly-His\textsubscript{6}, SA0620-His\textsubscript{6} and SA2097-His\textsubscript{6} were isolated from the growth medium fractions (Table 3).

Table 3 Overproduction of heterologous proteins

<table>
<thead>
<tr>
<th>Name; NCBI\textsuperscript{a}</th>
<th>Function</th>
<th>Domains\textsuperscript{b}</th>
<th>SP\textsuperscript{c}</th>
<th>Surface\textsuperscript{d}</th>
<th>Constructs</th>
<th>pI\textsuperscript{e}</th>
<th>kDa\textsuperscript{f}</th>
<th>Isolated amounts\textsuperscript{g}</th>
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</thead>
<tbody>
<tr>
<td>LytM; SA0265</td>
<td>Glycyl-glycyl endopeptidase</td>
<td>Y</td>
<td>Y (2, 3, 5, 6, 7)</td>
<td>pPA180:lytM::his\textsubscript{6}</td>
<td>6.37</td>
<td>33.1</td>
<td>2.7</td>
<td>11.0</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>pNG400:lytM::his\textsubscript{6}</td>
<td>9.10</td>
<td>57.1</td>
<td>4.0</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pPA224:lytM</td>
<td>9.54</td>
<td>51.0</td>
<td>4.5</td>
<td>nd</td>
</tr>
<tr>
<td>SA0620</td>
<td>SsaA-like putative autolysin</td>
<td>CHAP(C); LytM(N2)</td>
<td>Y</td>
<td>Y (6, 7)</td>
<td>pPA180:ssa620::his\textsubscript{6}</td>
<td>6.44</td>
<td>27.0</td>
<td>0.5</td>
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<td></td>
<td></td>
<td></td>
<td>pNG400:ssa620::his\textsubscript{6}</td>
<td>9.68</td>
<td>20.1</td>
<td>4.5</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pPA224:ssa620</td>
<td>10.02</td>
<td>44.1</td>
<td>4.0</td>
<td>nd</td>
</tr>
<tr>
<td>Nuc; SA0746</td>
<td>Thermonuclease</td>
<td>Y</td>
<td>Y (1, 4, 6, 7)</td>
<td>pPA180:nuc::his\textsubscript{6}</td>
<td>6.19</td>
<td>16.1</td>
<td>0.6</td>
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<td>pNG400:nuc::his\textsubscript{6}</td>
<td>9.55</td>
<td>40.1</td>
<td>4.0</td>
<td>nd</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>pPA224:nuc</td>
<td>8.62</td>
<td>91.7</td>
<td>24.9</td>
<td>nd</td>
</tr>
<tr>
<td>SsaA-h; SA2097</td>
<td>Hypothetical protein</td>
<td>CHAP(C)</td>
<td>Y</td>
<td>Y (1, 4, 5, 6, 7)</td>
<td>pPA180:ssa2097::his\textsubscript{6}</td>
<td>6.10</td>
<td>67.6</td>
<td>0.2</td>
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<td>pNG400:ssa2097::his\textsubscript{6}</td>
<td>8.62</td>
<td>91.7</td>
<td>24.9</td>
<td>nd</td>
</tr>
<tr>
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<td></td>
<td>pPA224:ssa2097</td>
<td>10.02</td>
<td>44.1</td>
<td>4.0</td>
<td>nd</td>
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<tr>
<td>Aly; SA2437</td>
<td>N-acetyluramyl-t-alanine mureinidase</td>
<td>CHAP(C)</td>
<td>Y</td>
<td>Y (1, 4, 5, 6, 7)</td>
<td>pPA180:aly::his\textsubscript{6}</td>
<td>6.19</td>
<td>16.1</td>
<td>0.6</td>
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<td>pNG400:aly::his\textsubscript{6}</td>
<td>9.55</td>
<td>40.1</td>
<td>4.0</td>
<td>nd</td>
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<td></td>
<td></td>
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<td>pPA224:aly</td>
<td>8.62</td>
<td>91.7</td>
<td>24.9</td>
<td>nd</td>
</tr>
<tr>
<td>SsaA; SA2356</td>
<td>Immunodominant antigen A, transglycosylase</td>
<td>Y</td>
<td>Y (1, 3, 4, 6, 5)</td>
<td>pPA180:ssa2356::his\textsubscript{6}</td>
<td>5.89</td>
<td>24.9</td>
<td>2.1</td>
<td>nd</td>
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</table>

\textsuperscript{a} SA number based on NCBI annotations of genome \textit{S. aureus} strain N315; \textsuperscript{b} the number and location (both indicated between brackets) of specific domains according the Pfam database (http://pfam.sanger.ac.uk/search); \textsuperscript{c} signal peptide (SP) according to SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/); \textsuperscript{d} surface location of protein according to \textsuperscript{(1)} (4); \textsuperscript{(2)} (43); \textsuperscript{(3)} (33); \textsuperscript{(4)} (44); \textsuperscript{(5)} (45); \textsuperscript{(6)} (34); \textsuperscript{(7)} (46); \textsuperscript{e} isoelectric point and molecular weight (f) of the secreted protein including the His-tag or PA-fusion; \textsuperscript{f} amount of protein (mg) isolated from 500 ml of culture; \textsuperscript{g} nd not determined.

Lastly, we investigated whether intracellularly or extracellularly produced and subsequently purified proteins were active. As shown in Fig. 8, the Nuc-His\textsubscript{6} purified from cells or the growth medium efficiently degraded chromosomal DNA of \textit{S. aureus} N315. The peptidoglycan-degrading activity of LytM-His\textsubscript{6} purified from cells or the growth medium was tested by incubation with cells of \textit{S. aureus} Newman. After 90 min incubation at 37°C, a 14.0% reduction of the \textit{OD}\textsubscript{600} was observed for the \textit{S. aureus} cells incubated with LytM-His\textsubscript{6}, isolated
from *L. lactis* cells. Similarly, a reduction of 16.4% in OD$_{600}$ was observed when LytM-His$_6$ purified from the *L. lactis* medium was used. In contrast, the OD$_{600}$ decreased merely by 6.2% in the absence of LytM-His$_6$. Activity of Aly-His$_6$ purified from *L. lactis* growth medium was enzymatically active, as visualized with zymograms using *M. lysodeikticus* as a substrate (Results not shown). From these results, we conclude that biological active His-tagged staphylococcal proteins can be efficiently isolated from overproducing *L. lactis* cells or the *L. lactis* growth medium using affinity chromatography. Notably, purification from the growth medium involves only a one-step procedure where the growth medium is directly used for metal affinity purification.

![Figure 8. Detection of Nuclease activity of purified Nuc-His$_6$. Chromosomal DNA of *S. aureus* N315 (1) was incubated for 5 min with commercial DNAse (2), Nuc-His$_6$ isolated from the cytoplasmic fraction of *L. lactis* (pPA180::nuc::his$_6$) (3) or the growth medium of *L. lactis* (pNG400::nuc::his$_6$) (4).](image)

**Discussion**

The present study was aimed at developing a pipeline for the expression and purification of tagged surface-exposed and secreted antigens from *S. aureus*. For this purpose, the *L. lactis* strain PA1001 was selected as an expression host that lacks both the major HtrA protease and the autolysin AcmA. The results show that all six tested *S. aureus* antigens were produced in *L. lactis*, either intracellularly or extracellularly, albeit to different levels. When provided with a C-terminal His-tag these antigens could be readily isolated using metal affinity
chromatography and, where tested, the purified proteins had retained their biological activity. Furthermore, our results show that *S. aureus* antigens can be expressed effectively in *L. lactis* with a PA-tag for cell surface display, or an AVI-tag for labeling with biotin.

In a previous study it was shown that the HtrA protease can interfere with efficient protein production in *L. lactis* (23). This view is supported by our observation that the *S. aureus* LytM-His₆ protein was subject to proteolysis in an HtrA-proficient strain. Nevertheless, the htrA mutation did not completely preclude cleavage of produced *S. aureus* antigens. This degradation was particularly evident for SA0620-His₆, but also in the case of Nuc-His₆ a minor degradation product was detectable by Western Blotting. At present, we do not know which protease is responsible for the observed cleavage, but it could be the cytoplasmic ClpP protease since ClpP was previously implicated in Nuc degradation (31).

Five of the investigated *S. aureus* antigens were expressed either with or without the signal peptide of the secreted *L. lactis* protein Usp45. Four of these five antigens, were efficiently secreted due to the presence of the Usp45 signal peptide. Only the secretion of SA0620-His₆ remained undetectable. In contrast, all five investigated antigens were produced intracellularly in absence of the Usp45 signal peptide. This included SA0620-His₆, which could subsequently be purified from the cells. The latter observation shows that for some *S. aureus* antigens, intracellular production may be an option if secretion cannot be achieved.

The *S. aureus* antigens Aly-His₆, LytM-His₆, Nuc-His₆ and SA2097-His₆ were successfully secreted using the Usp45 signal peptide. Nevertheless, substantial amounts of each of these proteins were retained in the respective producing cells. For Aly-His₆ and SA2097-His₆, this relates most likely to the presence of domains for non-covalent cell wall-binding. Specifically, the Aly and SA2097 proteins have so-called cysteine histidine dependent amidohydrolase/peptidase (CHAP) cell wall-binding domains (5,32), which may be responsible for cell retention. Notably, a C-terminal CHAP domain is also present in SA0620 and, moreover, this protein has two N-terminal LysM domains that also facilitate non-covalent cell wall-binding (5). It is thus very well conceivable that this protein is completely retained in the *L. lactis* cell wall by its CHAP and LysM domains after protein translocation from the cytoplasm. Furthermore, the
LytM and Nuc protein have a dual localization in *S. aureus*, being present both in the cell wall and the growth medium (33,34). It is therefore not surprising that this is also the case when these proteins are exported from the *L. lactis* cytoplasm using the Usp45 signal peptide. Yet, the precise cell wall-binding mechanism(s) of LytM and Nuc are currently enigmatic.

Importantly, the extracellular production of *S. aureus* antigens in *L. lactis* simplifies their isolation as exemplified with Aly-\text{His}_6, LytM-\text{His}_6, Nuc-\text{His}_6 and SA2097-\text{His}_6, which were all purified in one step from culture supernatants using metal affinity chromatography. For Aly-\text{His}_6, LytM-\text{His}_6 and Nuc-\text{His}_6, the yields were clearly higher when these proteins were purified from the growth medium than from the cells. Only in the case of SA2097-\text{His}_6, similar amounts of protein were isolated from cells or the growth medium but, clearly, the one-step purification from the growth medium was easier.

In conclusion, our present observations show that the non-lysing and proteolytically reduced *L. lactis* strain PA1001 is an excellent expression host for surface-bound and secreted antigens of *S. aureus*. While all tested antigens were produced and purified, for some antigens like SA0620 higher yields may be desirable. As was shown in previous studies this can potentially be achieved through the deletion of the gene for the major cytoplasmic protease ClpP (31), or deletion of the *ybdD* gene for a protein of unknown function, which was recently shown to enhance Nuc secretion by *L. lactis* (35). Alternatively, the secretion properties of *L. lactis* can potentially be enhanced by heterologous expression of the *B. subtilis* SecDF protein, a non-essential protein translocation machinery component that is absent from *L. lactis*, as was shown by Nouaille et al. (36). Lastly, it could be an option to improve the secretion of problematic antigens by mutating the signal sequence of Usp45, as was shown by Ng. and Sarkar [17], or by the use of alternative tags. Whether this will lead to gram per liter protein yields as obtained, for example, with *Bacillus* species is questionable. However, we believe that *L. lactis* will be suitable for producing sufficient amounts of protein for pilot studies and, if needed, bacilli could eventually be used for bulk production. However, this would then require mutated *Bacillus* strains with minimal protease and autolytic activity (37).
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Author’s Contributions
JN, DGAMK, TB, JMvD, GB conceived and designed the experiments. JN, DGAMK performed the experiments (DGAMK was responsible for protein purification). JN, DGAMK, JMvD, GB analyzed the data. TB, JMvD, GB contributed reagents, materials and analysis tools. JN, JMvD, GB wrote the manuscript. All authors have reviewed and approved the final manuscript.

References


