From the wound to the bench: exoproteome interplay between wound-colonizing Staphylococcus aureus strains and co-existing bacteria

Andrea N. García-Pérez, Anne de Jong, Sabryna Junker, Dörte Becher, Monika A. Chlebowicz, José C. Duipmans, Marcel F. Jonkman & Jan Maarten van Dijl

To cite this article: Andrea N. García-Pérez, Anne de Jong, Sabryna Junker, Dörte Becher, Monika A. Chlebowicz, José C. Duipmans, Marcel F. Jonkman & Jan Maarten van Dijl (2018) From the wound to the bench: exoproteome interplay between wound-colonizing Staphylococcus aureus strains and co-existing bacteria, Virulence, 9:1, 363-378, DOI: 10.1080/21505594.2017.1395129

© 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group

Accepted author version posted online: 13 Dec 2017. Published online: 01 Mar 2018.

Article views: 142

View Crossmark data

Submit your article to this journal

View related articles

Citing articles: 1 View citing articles
From the wound to the bench: exoproteome interplay between wound-colonizing *Staphylococcus aureus* strains and co-existing bacteria

Andrea N. García-Pérez, Anne de Jong, Sabryna Junker, Dörte Becher, Monika A. Chlebowicz, José C. Duipmans, Marcel F. Jonkman and Jan Maarten van Dijl

Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, Groningen, the Netherlands; Institute for Microbiology, Ernst-Moritz-Arndt Universität Greifswald, Friedrich-Ludwig-Jahn-Str. 15, Greifswald, Germany; Department of Dermatology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, RB Groningen, the Netherlands.

**ABSTRACT**

Wound-colonizing microorganisms can form complex and dynamic polymicrobial communities where pathogens and commensals may co-exist, cooperate or compete with each other. The present study was aimed at identifying possible interactions between different bacteria isolated from the same chronic wound of a patient with the genetic blistering disease epidermolysis bullosa (EB). Specifically, this involved two different isolates of the human pathogen *Staphylococcus aureus*, and isolates of *Bacillus thuringiensis* and *Klebsiella oxytoca*. Particular focus was attributed to interactions of *S. aureus* with the two other species, because of the high staphylococcal prevalence among chronic wounds. Intriguingly, upon co-cultivation, none of the wound isolates inhibited each other’s growth. Since the extracellular proteome of bacterial pathogens is a reservoir of virulence factors, the exoproteomes of the staphylococcal isolates in monoculture and co-culture with *B. thuringiensis* and *K. oxytoca* were characterized by Mass Spectrometry to explore the inherent relationships between these co-existing bacteria. This revealed a massive reduction in the number of staphylococcal exoproteins upon co-culturing with *K. oxytoca* or *B. thuringiensis*. Interestingly, this decrease was particularly evident for extracellular proteins with a predicted cytoplasmic localization, which were recently implicated in staphylococcal virulence and epidemiology. Furthermore, our exoproteome analysis uncovered potential cooperativity between the two different *S. aureus* isolates. Altogether, the observed exoproteome variations upon co-culturing are indicative of unprecedented adaptive mechanisms that set limits to the production of secreted staphylococcal virulence factors.

**Introduction**

Studies on pathogenic bacteria, either in vitro or in animal models, commonly address the respective bacteria as secluded species. Such studies do not consider the natural ecosystem of bacteria that colonize and invade the human body, which may happen in competition or cooperation with the existing microbiota and other pathogens. Furthermore, although bacteria-host interactions are the key to understanding the pathophysiology of infectious diseases, it is also important to scrutinize the parallel relationships between different bacterial species. This segment of microbial ecology may also elucidate how pathogens adjust their metabolism and other adaptive responses to survive and persist in the human body.

An example of a well-adapted bacterium associated with humans and animals, both as a commensal and as a pathogen, is *Staphylococcus aureus* [1, 2]. This bacterium has an exceptional ability to adapt and evolve swiftly into multidrug resistant lineages, as shown since the clinical introduction of penicillin and subsequently developed antibiotics [3, 4]. *S. aureus* is able to cause a wide range of afflictions ranging from impetigo and upper respiratory tract infections to osteomyelitis, endocarditis and sepsis. The fruitless attempts to develop a vaccine against *S. aureus* [5, 6], suggest the need for a better understanding of community interactions and the respective metabolic networks. This need is further underpinned by the high prevalence of infections caused by methicillin-resistant *S. aureus* (MRSA) in hospitals and communities worldwide [7, 8]. Nevertheless, the vast majority of molecular studies on the virulence of *S. aureus* have investigated this pathogen in isolation.
In the present study, we explored the possible interactions between two phenotypically different S. aureus isolates from a chronic wound of a patient with epidermolysis bullosa (EB) and two other bacterial species, Bacillus thuringiensis and Klebsiella oxytoca, isolated from the same wound. EB is a genetic blistering disease characterized by the development of chronic wounds upon simple mechanical trauma. It was previously shown that S. aureus is an important wound colonizer in patients with EB, where a patient can carry several S. aureus types in a single wound, and autoinoculation from the upper respiratory tract can occur [9–15]. Notably, next to S. aureus, also other bacteria are known to colonize the wounds of EB patients [16]. Considering these colonization characteristics and the high prevalence of S. aureus in the chronic wounds of patients with EB [15], we decided to study the interactions of S. aureus wound isolates with coexisting B. thuringiensis or K. oxytoca isolates. In particular, we focused attention on the extracellular proteomes (in short, exoproteomes) of these bacteria, because the exoproteome is a major reservoir of virulence factors [17].

B. thuringiensis is a Gram-positive, spore-forming aerobic bacterium that is commonly used as a safe bioinsecticide since it produces pore-forming proteins that are toxic for insect larvae [18]. It belongs to the same group as Bacillus anthracis and Bacillus cereus, the etiologic agents of anthrax and the “fried rice syndrome” (i.e. food poisoning), respectively [19, 20]. B. thuringiensis, unlike the other two, is not known as a major human pathogen [21]. However, it has been reported that this bacterium may be responsible for opportunistic pulmonary infections in susceptible subjects [22, 23]. On the other hand, the Gram-negative bacterium K. oxytoca is clearly regarded as an opportunistic pathogen. It is commonly found on mucosal surfaces of mammals, including the nasopharynx and colon of humans [24]. Klebsiella species are the second most common cause of nosocomial Gram-negative bacteremia after Escherichia coli, and they are often involved in urinary tract infections in risk group patients and neonatal sepsis [25–28].

Altogether, the present study was aimed at exploring the potentially synergistic or competitive relationships between S. aureus and two different classes of bacteria that are, in principle, capable of colonizing and invading the human body, thereby causing disease. Our specific objective was to unravel the bacterial response patterns using the exoproteome as a read-out. To this purpose, two different S. aureus isolates were, respectively, co-cultured with B. thuringiensis (Bt) or K. oxytoca (Ko) isolates from the same wound environment, and the exoproteomes of individually or co-cultured bacteria were analyzed by Mass Spectrometry (MS).

**Results**

**S. aureus grows unimpaired when co-cultured with B. thuringiensis or K. oxytoca on solid agar**

In a previous study, we applied replica plating of used wound dressings to assess the topography of distinct S. aureus types in chronic wounds of patients with EB [13]. Notably, this previous study was focused on S. aureus only, and it did not take into consideration other coexisting microbial species that are clearly evident upon replica plating of wound dressings (Fig. 1A). Such coexisting species were collected in the present study, using the dressing of a chronic wound from a patient with Junctional Epidermolysis Bullosa. This led to the identification of two S. aureus isolates with spa types t111 and t13595 that grew in close proximity to B. thuringiensis (Bt) and K. oxytoca (Ko) isolated from the same wound. A zone inhibition experiment was employed to assess possible competitive or bactericidal interactions between these four wound isolates. As shown for the S. aureus t111 and t13595 isolates in Fig. 1B, there was no growth inhibition detectable when, respectively, Bt or Ko were spotted onto blood agar plates that were confluent inoculated with S. aureus. The same was essentially observed when Bt was spotted onto a confluent inoculated plate with Ko. Additionally, B. subtilis strain 168 was chosen as control based on the known growth inhibition that B. subtilis 168 exerts on S. aureus due to production of the bacteriocin sublancin [29]. Indeed, the spotting of B. subtilis 168 onto the S. aureus cells caused a clear growth inhibition zone (Fig. 1B). Furthermore, it was noted that the growth of Bt and Ko occurred on top of the S. aureus spread. To confirm this observation, serial dilution experiments were performed with mixed cultures of S. aureus and Bt, or S. aureus and Ko. As marked by the arrows in Fig. 1, colonies of S. aureus were not only able to grow in the proximity of Bt and Ko colonies, but some were observed to touch or even grow on top of each other (Fig. 1C). This is fully consistent with the situation encountered in the wound environment from which the bacteria were isolated and where they thrive in close proximity (Fig. 1A).

**S. aureus isolates t111 and t13595 are highly related but not identical**

As a first step in the characterization of the selected S. aureus t111 and t13595 isolates, their antibiotic resistance profiles were determined. Both isolates were shown to be resistant to tetracycline, ciprofloxacin and fusidic acid. In addition, isolate t111 was found to be resistant to trimethoprim and sulfamethoxazole (Supplemental Table S1). These findings suggested that both isolates are
very similar, but not identical. This was confirmed by whole-genome sequencing, revealing that the respective core genomes differed only by 192 nucleotides. Genome sequencing further revealed that both S. aureus isolates belong to the sequence type 5 (ST5). Thus, the closest related reference genome is the one of S. aureus strain N315 which was, therefore, used for comparative genomic analyses and annotation.

Although there are not many differences in the core genomes of the t111 and t13595 isolates, a few of these differences are noteworthy. For example, the fnbA and fnbB genes of the t111 isolate were recombined into a single gene. Isolate t13595 contained a point mutation in agrA specifying the replacement of Gly148 with Asp, and this mutation was correlated with an agr negative phenotype (i.e. unlike the t111 isolate, the t13595 isolate displayed no α, β or δ-hemolysis). Frame shift mutations were also observed in various other genes of the t13595 isolate, including the mutL gene for a DNA mismatch repair protein, and genes such as sib, araC, geh and fnbA. For a more detailed comparison of the S. aureus isolates with respect to different mobile genetic elements, please refer to Supplemental Table S2.

**Total numbers of S. aureus exoproteins are reduced when S. aureus is co-cultured with Bt or Ko**

Since the S. aureus, Bt and Ko isolates addressed in this study were co-existent in the same wound environment and are capable of growing in close contact in an *in vitro* setting, we wanted to know whether and, if so, how they influence each other. To address this question, we selected a proteomics approach in which we focused our attention on the extracellular proteome. Further, to simulate the conditions in the human body, we selected the tissue culture medium RPMI as the growth medium for our studies, because S. aureus cells grown on RPMI or human plasma display very similar genome-wide transcript profiles [30]. Thus, the S. aureus isolates t111 and t13595 were cultured in three different conditions, namely in pure culture, or in combination with either Bt or Ko. As expected from the plating experiments, we observed only slight differences in the logarithmic growth rates of the mixed cultures as assessed by OD_{600} readings. For example, when S. aureus was co-cultured with Bt, the overall growth seemed slightly slower, while the opposite happened when S. aureus was co-cultured with Ko (Figure S1). This can be explained by the particular growth rates of the Bt and Ko isolates on RPMI, where the growth rate of Bt in monoculture is relatively slow, while that of Ko is relatively fast. Nevertheless, the overall growth patterns of the co-cultures were similar to those of the S. aureus pure cultures and, importantly, they were highly reproducible. Hence, in all the experiments, our reference for sampling was the S. aureus growth curve in monoculture.

To obtain an overview of the behavior of the cultures, we compared the total numbers of proteins detected by LC-MS/MS in all conditions (Fig. 2A,B) and verified the reproducibility of the biological replicates (Supplemental Figure S2). This analysis revealed clear differences between the pure and mixed cultures. Particularly, a total
of 64 extracellular proteins were detected in the *S. aureus* t111 monoculture, while in combination with Bt only 26 extracellular staphylococcal proteins were detected, and merely 16 when the t111 isolate was cultured with Ko. Essentially the same was observed for the exoproteome of the strain t13595, where 49 extracellular proteins were found in the monoculture, and only 29 and 12 in the co-cultures with Bt or Ko, respectively (Fig. 2A). We also assessed the numbers of extracellular proteins detected in the monocultures of Bt and Ko in comparison to the co-cultures. The results indicate a decrease of ~25% in the number of Ko proteins found in the co-cultures with *S. aureus* (Fig. 2B), but this effect was clearly not as prominent as the effect of Ko on the staphylococcal exoproteome. On the other hand, the number of exoproteins of Bt decreased only in the presence of the *S. aureus* t111 isolate but not in the presence of the *S. aureus* t1359 isolate (Fig. 2B). Of note, the exoprotein differences among the cultures were also reflected in the absolute amounts of protein detectable in the growth medium (Figure S3).

As a first approach to characterize the identified extracellular proteins, we assessed their predicted

![Figure 2](image-url)
Co-cultured S. aureus, B. thuringiensis and K. oxytoca isolates display species-specific exoproteome changes in terms of biological processes

Although both S. aureus isolates are closely related, particular variations in their exoproteomes were observed. For example, the core exoproteome of the t111 isolate included only 14 proteins (i.e. proteins constant in all cultures), while the core exoproteome of the t13595 isolate included only nine proteins (Fig. 3 and Table S3). Likewise, the biological processes in which these proteins are involved were also different. The core exoproteome of the t111 isolate was characterized by functions in peptidoglycan catabolic processes, pathogenesis and cobalamin transport, whereas the core exoproteome of the t13595 isolate is involved in cell redox homeostasis, cell wall organization, glucose metabolic processes, pathogenesis, cell adhesion and glycine betaine biosynthetic processes from choline.

Remarkably, there were not many staphylococcal proteins expressed uniquely in the co-cultures. In fact, there were no distinctive proteins detected in the co-cultures with Ko for both S. aureus isolates (Fig. 3A,B). For the co-cultures with Bt, we identified only the translocase subunit SecG as a distinctive feature in the growth medium of the t111 isolate, and an uncharacterized distinctive protein was identified in the growth medium of the t13595 isolate. In contrast, in the presence of the t111 or t13595 isolates, Ko displayed 9 and 8 unique extracellular proteins, respectively (Fig. 3D). The latter 9 proteins produced in the presence of the t111 isolate were related, by their gene ontologies, to chromosome condensation, regulation of transcription, the phosphoenolpyruvate-dependent sugar phosphotransferase system, and glycerol ether metabolic processes. Similarly, Bt displayed 18 distinctive extracellular proteins in the presence of the t13595 isolate (Fig. 3C). These proteins relate to iron-sulphur cluster assembly, protein transport, cell cycle, glucose and glycerol ether metabolic processes.

To further categorize the identified extracellular proteins, we classified them according to their function and categorized them in one of the following six groups: (1) cell envelope and cellular processes (including cell wall/membrane, transport/binding proteins and lipoproteins, signal transduction, membrane bioenergetics, motility and chemotaxis, protein secretion, cell division, cell adhesion, response to stress); (2) information pathways (including DNA restriction/modification and repair, transcription and regulation, ribosomal proteins, protein synthesis/modification/folding); (3) intermediary metabolism; (4) cell redox homeostasis, (5) virulence factors; and (6) unknown functions (see Supplemental Table S3).

In the diagrams of Fig. 4, the differences between the S. aureus t111 and t13595 isolates in terms of the functions of extracellular proteins are clearly evident; while the t111 isolate seems to be more virulent, the extracellular proteins of the t13595 are more involved in processes related to cell redox homeostasis and intermediary metabolism. Further, the core functions of the t13595 isolate appear more diverse than those of the t111 isolate. In the case of Ko, the overall picture indicates that this bacterium’s exoproteome is strongly involved in cell envelope and cellular processes, especially processes relating to the transport and binding of proteins and lipoproteins (Supplemental Figure S5A). For Bt, we observed that the response towards the co-cultured S. aureus isolates was considerably different. In particular, the extracellular Bt proteins related to many more different functions when Bt was co-cultured with the t13595 isolate than when Bt was co-cultured with the t111 isolate. Interestingly, like the S. aureus t13595 isolate co-cultured with Bt, Bt also exhibited many extracellular proteins related to redox homeostasis when co-cultured with the t13595 isolate (Supplementary Figure S5B).
Particular exoproteins of *S. aureus*, *K. oxytoca* and *B. thuringiensis* show quantitative changes in mono- or co-cultures

Conceivably not all the differences in the exoproteome composition of mono- and co-cultured *S. aureus*, *Ko* and *Bt* are absolute, but also quantitative differences may exist for particular exoproteins. Therefore, we comparatively assessed significant fold-changes in the exoproteins detected in mono- and co-cultures by mapping their spectral counts from all replicates using volcano plots (Supplemental Figure S6). The results of this analysis, presented in Table 1, indicate that there were 11 exoproteins significantly ‘upregulated’ in the *S. aureus* monocultures compared to the co-cultures with *Bt* or *Ko*. These included *S. aureus* proteins, such as elongation factor Ts, triosephosphate isomerase, thioredoxin reductase, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase and a

---

*Figure 3.* Relationships among cultures. The diagrams show the number of proteins identified in all cultures and those proteins shared in the different conditions. (A) Total number of *S. aureus* t111 proteins in monoculture (11) and in co-culture with *B. thuringiensis* (11B) or *K. oxytoca* (11K); (B) Total number of *S. aureus* t13595 proteins in monoculture (13) and in co-culture with *B. thuringiensis* (13B) or *K. oxytoca* (13K); (C) Total number of *B. thuringiensis* proteins in monoculture (B) and in co-culture with *S. aureus* t111 (B11) or *S. aureus* t13595 (B13); (D) Total number of *K. oxytoca* proteins in monoculture (K) and in co-culture with *S. aureus* t111 (K11) or *S. aureus* t13595 (K13); (E) Total number of *S. aureus* proteins in monoculture (11) or (13) and in co-culture (11+13).
secreted β-lactamase. In the case of Ko, 22 exoproteins were present in significantly different amounts when mono and co-cultures were compared. Here, 17 exoproteins of Ko were significantly upregulated in the monocultures, whereas 5 other exoproteins of Ko were significantly upregulated in the co-cultures with S. aureus. The latter proteins are mostly involved in carbohydrate metabolism. Also in the case of Bt, 22 exoproteins were

Figure 4. Overview of the predicted functions of the exoproteins found in all staphylococcal cultures. Diagram (A) depicts only S. aureus t111 proteins. The area on the top shows the proteins detected only in monoculture, while the lower left area shows a single protein detected when t111 was co-cultured with B. thuringiensis. Likewise, diagram (B) shows exclusively the proteins that belong to S. aureus t13595 in monoculture and co-culture with B. thuringiensis or K. oxytoca. Diagram (C) depicts the proteins of S. aureus t111 and t13595 grown in monoculture in the bottom areas, whilst the upper area shows the proteins identified upon co-culturing both S. aureus isolates. In the latter area, the circles are represented as pie charts. The charts show the percentage of proteins that belong to each isolate. For the unknown proteins (in grey), 50% belong to t111, 37% to t13595, and 12.5% to both of them; for the cell envelope proteins (blue) 67% belong to t111 and 33% to both isolates; for proteins involved in intermediary metabolism (green), 25% belong to t111 and 75% to t13595; and for information pathways proteins (yellow), 50% belong to t111 and 50% to both isolates.
Table 1. Summary of differentially detected extracellular proteins among the different cultures. Volcano plots (Supplemental Figure S6) displayed P values (-log₁₀) versus fold changes (log₂) of the normalized spectral counts. Folds ≤1 represent proteins that were significantly up-regulated in the co-cultures and folds >1 represent proteins that were significantly up-regulated in monoculture.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Function / Gene ontology</th>
<th>Fold</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>Beta-lactamase</td>
<td>Hydrolysis of beta-lactamic ring</td>
<td>12.81</td>
<td>0.041</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Phosphocarrier protein HPr</td>
<td>Phosphoenolpyruvate-dependent sugar phosphotransferase system. Serine/threonine kinase activity. Regulation of transcription, DNA-templated</td>
<td>6.61</td>
<td>0.009</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Fructose-bisphosphate aldolase class 1</td>
<td>Glycolytic process</td>
<td>12.89</td>
<td>0.042</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Elongation factor Ts</td>
<td>Binds to aminoacyl-tRNA. Protein biosynthesis</td>
<td>29</td>
<td>0.034</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Triosephosphate isomerase</td>
<td>Gluconeogenesis. Glycolytic process. Pentose-phosphate shunt</td>
<td>18</td>
<td>0.038</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2,3-bisphosphoglycerate-dependent phosphoglycerate mutase</td>
<td>Gluconeogenesis. Glycolytic process</td>
<td>16.80</td>
<td>0.048</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Cysteine synthase</td>
<td>Cysteine biosynthetic process from serine</td>
<td>13</td>
<td>0.045</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Phosphate acetyltransferase</td>
<td>Acetyl-CoA biosynthetic process</td>
<td>15.60</td>
<td>0.039</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Thioredoxin reductase</td>
<td>Removal of superoxide radicals</td>
<td>17</td>
<td>0.040</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Putative universal stress protein SSP1056</td>
<td>Response to stress</td>
<td>13</td>
<td>0.046</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>D-alanine-poly(phosphoribitol) ligase subunit 2</td>
<td>Cell wall organization. Lipoteichoic acid biosynthetic process. Regulation of cell shape</td>
<td>6</td>
<td>0.039</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Uncharacterized protein</td>
<td>Unknown</td>
<td>2.68</td>
<td>0.003</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Colicin I receptor</td>
<td>Bacteriocin transport. Iron assimilation. Siderophore transmembrane activity</td>
<td>1.63</td>
<td>0.036</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Glucose metabolic process. Glycolytic process</td>
<td>0.33</td>
<td>0.044</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>TonB-dependent hemin, ferriochrome receptor</td>
<td>Transporter activity</td>
<td>1.79</td>
<td>0.024</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Glutathione-binding protein GsbB</td>
<td>ABC transporter complex (periplasmic space)</td>
<td>1.26</td>
<td>0.043</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Phosphoglycerate kinase</td>
<td>ATP binding. Glycolytic process</td>
<td>0.37</td>
<td>0.030</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Glycine betaine-binding periplasmic protein</td>
<td>Transporter activity</td>
<td>1.81</td>
<td>0.010</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Protein ToB</td>
<td>Bacteriocin transport. Uptake of colicins (group A)</td>
<td>1.50</td>
<td>0.029</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Leucine-specific-binding protein</td>
<td>L-alpha-amino acid transmembrane transport</td>
<td>2.16</td>
<td>0.002</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Pyruvate kinase</td>
<td>Glycolysis. Magnesium, potassium and ATP-binding</td>
<td>0.23</td>
<td>0.038</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Glutamine-binding periplasmic protein</td>
<td>Amino acid transport.</td>
<td>1.49</td>
<td>0.040</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>6-phosphogluconolactonase</td>
<td>Pentose-phosphate pathway</td>
<td>0.41</td>
<td>0.020</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Osmotically-inducible lipoprotein E</td>
<td>Possibly involved in maintaining the structural integrity of the cell envelope</td>
<td>4.75</td>
<td>0.002</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Periplasmic serine endopeptidase DegP</td>
<td>Degrades transiently denatured and unfolded proteins accumulated in the periplasm in stress conditions</td>
<td>4.86</td>
<td>0.002</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Uncharacterized protein</td>
<td>Unknown</td>
<td>10</td>
<td>0.004</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Periplasmic trehalase</td>
<td>Cellular hyperosmotic response</td>
<td>3.60</td>
<td>0.049</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Uncharacterized protein</td>
<td>Unknown</td>
<td>6.67</td>
<td>0.011</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Uncharacterized protein</td>
<td>Unknown</td>
<td>0.13</td>
<td>0.031</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Peptidoglycan-associated lipoprotein</td>
<td>Possibly bacterial envelope integrity</td>
<td>3.20</td>
<td>0.019</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Chaperone protein MrkB</td>
<td>Cell wall organization. Chaperone-mediated protein folding. Pilus organization.</td>
<td>5.33</td>
<td>0.045</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>MltA-interacting protein</td>
<td>Peptidoglycan biosynthetic process</td>
<td>12.89</td>
<td>0.042</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>Thiamine-binding periplasmic protein</td>
<td>ATP binding. Thiamine transport</td>
<td>8</td>
<td>0.010</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Uncharacterized protein</td>
<td>Unknown</td>
<td>14.61</td>
<td>0.001</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Sulfatase</td>
<td>Sulphuric ester hydrolase activity</td>
<td>3.59</td>
<td>0.008</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Internalin</td>
<td>The function of this domain is not clear</td>
<td>4.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Endopeptidase lytE, putative lytE</td>
<td>The function of this domain is not clear</td>
<td>2.08</td>
<td>0.023</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Peptidase M4 thermolysin</td>
<td>The function of this domain is not clear</td>
<td>2.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Enolase</td>
<td>Glycolytic process</td>
<td>4.44</td>
<td>0.043</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Bacillolysin</td>
<td>Extracellular zinc metalloprotease</td>
<td>6.61</td>
<td>0.009</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Extracellular solute-binding protein family 5</td>
<td>The function of this domain is not clear</td>
<td>3.37</td>
<td>0.005</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>VanW</td>
<td>Vancomycin resistant protein W. The function is not clear</td>
<td>2.80</td>
<td>0.045</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Uncharacterized protein</td>
<td>Unknown</td>
<td>3.76</td>
<td>0.014</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Oligopeptide transporter, periplasmic-binding protein</td>
<td>ABC transporter complex</td>
<td>17</td>
<td>0.001</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Periplasmic component of efflux system</td>
<td>Transmembrane transport</td>
<td>18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Camelysin Metallo peptidase MEROPS family M73</td>
<td>The function of this domain is not clear</td>
<td>7.33</td>
<td>0.031</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Flagellar hook-associated protein 3</td>
<td>Bacterial-type flagellum-dependent cell motility</td>
<td>11</td>
<td>0.014</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Uncharacterized protein</td>
<td>Unknown</td>
<td>10</td>
<td>0.035</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Uncharacterized protein</td>
<td>Unknown</td>
<td>8</td>
<td>0.010</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Foldase protein PrsA 2</td>
<td>Protein folding</td>
<td>9</td>
<td>0.008</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Glucose-specific phosphotransferase enzyme IIA component</td>
<td>Phosphoenolpyruvate-dependent sugar phosphotransferase system</td>
<td>0.19</td>
<td>0.030</td>
</tr>
</tbody>
</table>
present in significantly different amounts when mono and co-cultures were compared (Table 1). Interestingly, 21 of these Bt exoproteins were significantly ‘upregulated’ in the monocultures compared to the co-cultures with S. aureus. The functions of these proteins were rather diverse, including the cell wall hydrolase LytE, three different proteases, and the protein folding catalyst PrsA.

Discussion

Our present co-culturing approach represents an opportunity to study changes in protein diversity as a result of inter-bacterial relationships that can, to a certain extent, reflect the natural situation in a wound. In principle, the in vitro growth characteristics of S. aureus colonies over B. thuringiensis or K. oxytoca colonies suggest adaptive mechanisms for all species. From the proteome perspective, one mechanism that stands out is the depletion of staphylococcal cytoplasmic proteins from the exoproteomes of co-cultures upon co-culture with Bt or Ko. Proteomic analysis also revealed two uniquely detectable exoproteins in the co-cultures with Bt. The exoprotein found in the co-culture of S. aureus t111+Bt was SecG, a subunit of the heterotrimeric complex SecYEG. This complex serves as a membrane channel for translocation of secretory proteins in an unfolded state [31]. In S. aureus, SecG is of importance since secG deletion mutants displayed impaired secretion of several wall-bound proteins and abundant exoproteins [32]. Despite its known function in protein secretion, the reason for the unique detection of SecG in the t111-Bt exoproteomes is presently not clear. Additionally, in S. aureus t13595+Bt co-cultures an uncharacterized protein that resembles a control factor of the competence regulator ComK, named YlbF/YmcA, was found. This protein is known to be involved in biofilm formation by other Gram-positive and archaean organisms. Further characterization of these two proteins might help us to understand why they are present in the exoproteome when S. aureus is co-cultured with Bt.

While the exoproteome of S. aureus in monoculture was previously found to contain numerous typically cytoplasmic proteins [17, 33–38], this is not an exclusive trait of S. aureus. The exoproteomes of other Gram-positive bacteria, such as B. subtilis, Clostridium difficile, Corynebacterium diphtheria, Group A Streptococcus, Mycobacterium tuberculosis and Streptomyces lividans, but also Gram-negative bacteria like Porphyromonas gingivalis, have been associated with high numbers of such typically cytoplasmic proteins.[36, 39–43] In B. subtilis, this observation has been explained by a combination of high cell lysis propensity, increased cell wall turnover and/or autolysin activity [44]. It has also been proposed that the ‘liberation’ of typical cytoplasmic proteins that lack the prototype signal peptides of proteins exported via the general Sec pathway for protein secretion could be facilitated by as yet unidentified alternative pathways, also referred to as ‘non-classical secretion systems’ [45–50]. For example, it is conceivable that, besides the well-characterized endolysins, the expression of prophage-encoded holins or holin-like proteins might have a role in the liberation of otherwise cytosolic proteins [42,50]. The release of cytosolic proteins due to the large pores formed by holins is still somewhat debated, since it has not been extensively studied in Gram-positive organisms. Nevertheless, the presence of additional holins could, thus, at least partially explain the elevated number of cytoplasmic proteins found in the growth medium of the S. aureus t13595 isolate compared to the t111 isolate. Yet, it has been shown for B. subtilis that engineered strains lacking all prophages still release typical cytoplasmic proteins into their growth medium [52]. Clearly, for the bacterial isolates investigated in the present study, it remains to be defined which particular mechanisms contribute, individually or synergistically, to the observed non-classical secretion phenomenon and to what extent.

Interestingly, the common denominator between the presently identified S. aureus exoproteome and that of its ‘wound mates’ is the presence of housekeeping enzymes, particularly glycolytic enzymes. For instance, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) found in the S. aureus t13595, Bt and Ko cultures, has also been located on the surface of other pathogenic bacteria like group A streptococci, and enteropathogenic E. coli where it can serve either as a binding protein or as signal transduction initiator in epithelial cells [53–55]. Cell-wall-associated proteins like enolase (displayed in the same cultures) have been reported to bind to plasminogen both in Gram-positive (Streptococcus pneumoniae) and Gram-negative (Actinobacillus actinomycetemcomitans) bacteria [56, 57]. Other multifunctional “moonlighting” proteins like fructose-bisphosphate aldolase, identified in S. aureus t111 cultures, are recognized to play a role in adhesion to mammalian cells and binding of human plasminogen by several bacterial species, like S. pneumoniae, M. tuberculosis, and Neisseria meningitidis [58]. Similarly, the elongation factor (EF) Tu, a guanosine nucleotide binding protein important for protein synthesis, was detected on the surface of Mycoplasma pneumoniae and Lactobacillus johnsonii, where it was involved in fibronectin- and mucin-binding, respectively [59, 60]. Furthermore, an immunoproteomic study of bacteremia showed that six housekeeping enzymes and EF-Tu were linked to leukopenia caused by Klebsiella pneumonia [61]. Altogether, these findings imply that proteins, which were originally considered to be restricted to the
cytoplasm, may also be important for virulence enhancement and invasive disease [50, 62]. Indeed, for *S. aureus*, such proteins have previously been implicated in virulence, epidemiology and intracellular survival [46–48, 50, 63].

By tracking the *S. aureus*-specific exoproteome, we observed that especially the cytoplasmic protein fractions were depleted from the exoproteome in co-cultures. This suggests that there might be either an altered localization, decreased cell lysis, enhanced proteolysis or consumption of these proteins by the other organism in the respective co-culture. In the literature, a loss of the cytoplasmic exoproteome fraction was recently associated to the latter. When studying marine bacteria in mixed cultures, Christie-Oleza et al. showed that extracellular cytoplasmic proteins from *Synechococcus* are notably reduced when *Roseobacter* strains are present [64]. Their study also reported an abundance of active transporters and flagellar proteins of *Roseobacter* during co-culturing. Such a situation could potentially also apply to *K. oxytoca* and *B. thuringiensis* as the present data suggests that, when grown in a nutrient-limited environment, these bacteria might act as scavengers of carbon and nitrogen sources, particularly by degrading and assimilating extracellular staphylococcal proteins. If so, this would suggest that, at least in a closed system like the one we established *in vitro*, nothing goes to waste and that the carbon and nitrogen fixing characteristics of Bt and Ko are perhaps a distinguishable reflection of short-term interspecies interactions. Nevertheless, we cannot exclude the possibility that there could also be less *S. aureus* cell lysis during co-culture. One way to verify the idea that Bt and Ko consume extracellular proteins of *S. aureus*, may involve the metabolic labeling of *S. aureus* proteins with particular isotopes, providing these proteins to Bt or Ko cultures, and investigating the incorporation of the isotopes into Bt or Ko proteins or metabolites. Likewise, interactions between *S. aureus* and Bt or Ko could be investigated by transcriptomics and metabolomics. Irrespective of the molecular background of our observations, the depletion of *S. aureus* proteins from the exoproteome upon co-culturing has an important implication for the virulence of this bacterium as exoproteins should be considered as the prime reservoir of virulence factors [17, 50].

Beyond the known proteome heterogeneity [33, 34, 37], and the genetic diversity of the colonizing staphylococcal strains [15, 65], we found a *mutL* mutation indicative of impaired mismatch repair and other hypermutable traits in our *S. aureus* t13595 isolate [66]. This finding allows us to place the relationships between the investigated *S. aureus* isolates in a broader perspective, where it is of note that the presently investigated bacterial isolates from an EB patient were obtained upon one-time sampling of a >8 months existing chronic wound. Importantly, it has been hypothesized that hypermutable strains play roles in species persistence and chronic infection [67, 68] and they could perhaps also favor the development of antibiotic resistance [69–71]. In the context of the long-term colonization of chronic wounds of EB patients by *S. aureus*, the influence of mutations on the intra-species heterogeneity should be considered in view of microbial social behavior and the consequences for fitness that particular mutations may provide [72]. Interestingly, with respect to the observed proteomic profiles, the *S. aureus* t111 isolate seems to foster a committed production of metabolically costly siderophores, virulence factors and cell adhesion molecules, while the t13595 isolate shows a more ‘recalcitrant’ performance focusing only on its metabolism and homeostasis. Moreover, during co-culturing of the t111 and t13595 isolates, we did not observe any protein overexpression, contrary to what was observed for the monocultures. This outcome is suggestive of a cooperative behavior. Therefore, it is tempting to hypothesize that the genetic variability as displayed by the ‘selfish’ t13595 isolate may be beneficial for the spread or persistence of *S. aureus* as a wound-colonizing species [73].

To the best of our knowledge, our research represents the first explanatory analysis of *S. aureus* exoproteomic shifts while in co-culture with wound-coexisting bacteria. Collectively, our findings point out the importance of studying bacteria in a broader biological context. It is evident that, for example, the growth rates of the four isolates explored here are not necessarily the same, and therefore it is not possible to compare the exoproteome of all investigated isolates at exactly the same growth stage. However, this situation is also true in a wound environment where not all co-existing microbes are completely synchronized or exist in equal numbers. Hence, the present data should be interpreted as one of many possible scenarios in which bacteria interact with each other with no apparent antagonistic relationships.

Because of the complexity of the wound environment and the peculiarities of each wound, it is difficult to establish a standard definition of the nutrients present in a wound. For example, temperature in a wound can influence local vasodilation and therefore the availability of nutrients. Furthermore, the biomolecules are primarily at the disposal of host cells rather than bacteria that have to compete for the nutrient restriction [74]. In the present investigations, we have presented a scenario that simulates the conditions in human plasma, which relies on bacteria cultivated under shaking and not static conditions. Accordingly, the present culture conditions may not exactly reflect the situation in chronic wounds of an EB patient. However, it seems reasonable to assume that
in the wound environment, cytosolic bacterial proteins could represent an important foundation for microbiome cross talk and homeostasis. Altogether, our experiments introduce an attractive avenue to explore possible pre- and probiotic targets, such as quorum sensing- or quorum quenching-related factors or bacterial growth factors that can either hinder the invasion by pathogenic bacteria, or enhance the presence of non-pathogenic microbes that may prevent infections and could even enhance wound healing.

### Material and methods

**Ethical approval**

The local medical ethics committee (METc) of the University Medical Center Groningen (UMCG) approved of the collection of non-invasive samples from patients with EB on the basis of obtained written informed patient consent. The experiments were conducted in accordance with the guidelines of the Declaration of Helsinki. All samples were anonymized.

**Bacterial isolation, identification and culture conditions**

The used bandage from a chronic wound of a Dutch patient with Junctional Epidermolysis Bullosa was collected. The bandage was replica-plated with gentle pressure for 10 seconds onto CLED agar (Oxoid) using bioassay plates (245 mm; Nunc). After 48 h of incubation at 37°C, colonies were selected and replated onto blood agar plates for identification. Bacterial colonies were identified by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS, using a MALDI Biotyper® (Bruker Corporation, Billerica, USA) as previously described [75]. All isolates were stored with 10% glycerol at −80°C.

To investigate whether the studied bacteria secrete compounds that interfere with each other’s growth, blood agar plates were spread with pure overnight cultures of *S. aureus* isolates with an optical density at 600 nm (OD₆₀₀) of 0.5 to obtain a confluent lawn of cells. Then, after 5 min, 10 µL aliquots of pure overnight cultures of *B. thuringiensis*, *K. oxytoca* or *Bacillus subtilis* 168 (OD₆₀₀ 0.5) were applied to the center of the agar plate. For viable plate counting and the preparation of extracellular protein extracts, bacterial strains were grown overnight on Tryptic Soy Broth (TSB) under vigorous shaking at 37°C. The cultures were then diluted to an OD₆₀₀ of 0.05 in pre-warmed RPMI medium without phenol red (GE Healthcare). Cultivation continued in a water bath under constant shaking (80–85 rpm) at 37°C until cultures reached an OD₆₀₀ of ±0.5. Main cultures were again diluted in 120 mL pre-warmed RPMI medium under the same conditions. Monocultures were started with an initial OD₆₀₀ of 0.05 while co-cultures were inoculated with an OD₆₀₀ of 0.025 of each isolate to a total of 0.05. Given that an OD₆₀₀ of 0.5 contained a total of ~200 × 10⁶ colony forming units per mL (CFU/mL) for t111, t13595 and Ko, and ~15 × 10⁶ CFU/mL for Bt, inoculated cultures corresponded to ~20 × 10⁶ CFU/mL for t111, t13595 and Ko and to ~1.5 × 10⁶ CFU/mL for Bt.

For proteome analyses and determination of CFU/mL, samples were collected at 90 min within the stationary growth phase. The ratios of different bacteria sampled from the co-cultures remained nearly the same with the exception of the co-cultures of *S. aureus* and *K. oxytoca*, where *K. oxytoca* grew 1.5 times faster than t111 and 1.7 times faster than t13595.

**Genomic DNA extraction, genome sequencing and analysis**

Libraries for whole genome sequencing were prepared from *S. aureus* t111, t13595, *B. thuringiensis*, and *K. oxytoca* DNA with the Nextera XT v2 kit, and run on Illumina next generation sequencing platforms (MiSeq) according to the manufacturers’ instructions (Illumina, San Diego, CA, USA). Read data for the study isolates have been deposited in the National Center for Biotechnology Information (NCBI) under accession number SRP094393. The respective .fastq files were submitted for de novo sequence assembly using CLC Genomics Workbench v7.0.4 (CLC bio A/S, Aarhus, Denmark) after quality trimming (Qs > 28) with optimal word sizes based on the maximum N50 value. De novo assembled contigs of the *S. aureus* isolates were imported into the Seqsphere+ software version 1.0 (Ridom GmbH, Würzburg, Germany) for in silico multi-locus sequence typing (MLST) and spa typing as previously described [76, 77]. Automatic genome annotation was done on the rapid annotation using subsystem technology (RAST) server 2.0 for all the genomes [78, 79]. De novo assembled genomes of sequenced isolates were queried against the closest related reference genome of *S. aureus* strain N315 (GenBank: NC_007795.1), *B. thuringiensis* serovar konkukian str. 97–27 (GenBank: NC_017200), and *K. oxytoca* strain E718 (GenBank: NC_018106) using blastN in the WebACT comparison tool (http://www.webact.org/WebACT/prebuilt#) and analyzed in detail by the Artemis Comparison Tool (ACT) software [80, 81]. Similarity matches were filtered based on their length (100 kb segments) and percentage similarity scores, and only the filtered hits with at least 80%
sequence similarity were then displayed by ACT (e-value of 10.00000) and analyzed in detail.

**Preparation of protein extracts**

All samples were collected in duplicate according to the growth curves of the *S. aureus* isolates after 90 min of stationary growth. Cells were removed by centrifugation (10 min, 8000 x g, 4°C) from 2 mL culture aliquots and the supernatant was subsequently filtrated with a 0.22 µm filter (GE Healthcare Systems, Little Chalfont, United Kingdom). Proteins were precipitated with 10% w/v TCA on ice at 4°C overnight. The precipitates were harvested by centrifugation (20 min, 8000 x g, 4°C) and washed with ice-cold acetone. The protein pellets were dried at room temperature and stored at −20°C until further use. Total protein concentration was determined with the Pierce BCA protein quantification assay (ThermoFisher Scientific). Protein pellets were suspended in 50 mM ammonium bicarbonate buffer (Fluka, Buchs, Switzerland). As reducing agent, 10 mM dithiothreitol (Duchefa Biochemie, Haarlem, the Netherlands) was used for 30 min, and as alkylating agent, 10 mM iodoacetamide (Sigma-Aldrich, St. Louis, USA) was used for 30 min in the dark. Further, overnight incubation with 80 ng of trypsin (Promega, Madison, USA) at 37°C was followed by the addition of 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, St. Louis, USA) to end the digestive reaction. The samples were desalted by Zip-Tip purification (Millipore, Billerica, USA) as previously described by Dreisbach et al. [82]. The final eluates were concentrated using a vacuum centrifuge (Eppendorf, Hamburg, Germany) and stored at 4°C until further use.

**Mass spectrometry and data analysis**

Tryptic peptides were separated by reverse phase liquid chromatography (LC). LC-MS/MS analyses were performed using a nanoACQUITY UPLC system (Waters, Milford, MA) coupled to an LTQ XL Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) creating an electro spray ionization through a Picotip Emitter (SilicaTip, FS360-20-10 Coating P200P, New Objective). Specifically, the peptides were loaded onto a trap column (Symmetry C18, 5 µm, 180 µm inner diameter × 20 mm, Waters). Elution was performed onto an analytical column (BEH130 C18, 1.7 µm, 100 µm inner diameter × 100 mm, Waters) by a binary gradient of buffer A (0.1% (v/v) acetic acid) and B (0.1% (v/v) acetic acid in acetonitrile) over a period of 80 min with a flow rate of 400 nL min⁻¹. A stepped gradient was applied.

For MS/MS analysis a full survey scan was recorded in the Orbitrap (m/z 300–2000) with a resolution of 30,000. The five most abundant precursor ions from each survey scan were consecutively isolated in the LTQ XL and fragmented via collision induced dissociation (CID). Precursors were dynamically excluded for 30 s and unassigned charge states as well as singly charged ions were rejected [83]. Internal calibration was applied (lock mass 445.12005 m/z). Database searching was done with Sorcerer-SEQUENT 4 (Sage-N Research, Milpitas, USA). After extraction from the raw files, “.dta files were searched with Sequest against a concatenated target-pseudoreversed decoy database with a set of common laboratory contaminants. The target database was the Uniprot reference database (downloaded on September 2014) of B. thuringiensis (5815 protein hits), K. oxytoca (5663 protein hits), S. aureus t111 (2574 protein hits) and S. aureus t13595 (2625 protein hits). The “.out files were compiled with Scaffold 4 (version Scaffold_4.4.1, Proteome Software Inc., Portland, OR). Protein identification criteria were carried out as specified by Hempel et al., [83], but with the following XCorr values: ≥ 2.2, 3.3, 3.75 for doubly, triply and higher charged peptides. To allow protein abundance comparisons between samples, Scaffold MS/MS data were normalized by adjusting the sum of the selected quantitative values for all proteins in the list within each MS sample to a common value: the average of the sums of all MS samples present in the experiment. This was achieved by applying a scaling factor for each sample to each protein or protein group adjusting in this way the normalized values for calculating the fold-changes in each protein among the monoculture samples versus the co-culture isolates after 90 min of stationary growth. Cells were removed by centrifugation (10 min, 8000 x g, 4°C) from 2 mL culture aliquots and the supernatant was subsequently filtrated with a 0.22 µm filter (GE Healthcare Systems, Little Chalfont, United Kingdom). Proteins were precipitated with 10% w/v TCA on ice at 4°C overnight. The precipitates were harvested by centrifugation (20 min, 8000 x g, 4°C) and washed with ice-cold acetone. The protein pellets were dried at room temperature and stored at −20°C until further use. Total protein concentration was determined with the Pierce BCA protein quantification assay (ThermoFisher Scientific). Protein pellets were suspended in 50 mM ammonium bicarbonate buffer (Fluka, Buchs, Switzerland). As reducing agent, 10 mM dithiothreitol (Duchefa Biochemie, Haarlem, the Netherlands) was used for 30 min, and as alkylating agent, 10 mM iodoacetamide (Sigma-Aldrich, St. Louis, USA) was used for 30 min in the dark. Further, overnight incubation with 80 ng of trypsin (Promega, Madison, USA) at 37°C was followed by the addition of 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, St. Louis, USA) to end the digestive reaction. The samples were desalted by Zip-Tip purification (Millipore, Billerica, USA) as previously described by Dreisbach et al. [82]. The final eluates were concentrated using a vacuum centrifuge (Eppendorf, Hamburg, Germany) and stored at 4°C until further use.

**Bioinformatic analyses**

Subcellular protein localization was predicted with TMHMM (version 2.0) [85, 86], SignalP (version 4.1) [87], LipoP (version 4.1) [88], PSORTb (version 3.0) [89], and ClusSub-P (version 2.18.3) [90]. Proteins with ambiguous predictions were manually curated by inspection of their individual sequences with InterProScan 5 (version 49.0) [91]. For interpretation and visualization of biologically relevant protein functions, the gene ontology (GO) terms identified for the investigated exoproteomes were imported into the freely available online Revigo software (http://revigo.irb.hr/) [92].
Biological and chemical safety

*S. aureus* and *K. oxytoca* are biosafety level 2 (BSL-2) microbiological agents and were accordingly handled following appropriate safety procedures. All experiments involving live bacteria and chemical manipulations of bacterial protein extracts were performed under appropriate containment conditions. All chemicals and reagents used in this study were handled according to the local guidelines for safe usage and protection of the environment.

Disclosure of potential conflicts of interest

The authors declare that they have no financial and non-financial competing interests in relation to the documented research.

Acknowledgments

We thank Corinna Glasner, Andreas Otto, Tim Stobernack, Eleni Tsompanidou and Kai Zhou for helpful discussions.

Ethics

The present research has no particular ethical implications.

Funding

This work was supported by the Graduate School of Medical Sciences of the University of Groningen, CoNaCyT, the Eleven Flowers Fund and the Ubbo Emmius Fund [to A.N.G.P. and J. M.v.D.], and by the Deutsche Forschungsgemeinschaft Grants GRK1870 and SFB/TRR 34 [to D.B.].

References


