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Enteric YaiW Is a Surface Exposed Outer Membrane Lipoprotein that Affects Sensitivity to an Antimicrobial Peptide

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ABSTRACT

*yaiW* is a previously uncharacterized gene found in enteric bacteria that is of particular interest because it is located adjacent to the *sbmA* gene, whose *bacA* ortholog is required for *Sinorhizobium meliloti* symbiosis and *Brucella abortus* pathogenesis. We show that *yaiW* is co-transcribed with *sbmA* in *Escherichia coli* and *Salmonella enterica* serovars Typhi and Typhimurium strains. We present evidence that the YaiW is a palmitate-modified surface exposed outer membrane lipoprotein. Since BacA function affects the very long chain fatty acid (VLCFA) modification of *S. meliloti* and *B. abortus* lipid A, we tested whether SbmA function might affect either the fatty acid modification of the YaiW lipoprotein or the fatty acid modification of enteric lipid A, but found that it did not. Interestingly, we did observe that *E. coli* SbmA suppresses deficiencies in the VLCFA modification of the LPS of an *S. meliloti bacA* mutant despite the absence of VLCFA in *E. coli*. Finally, we found that both YaiW and SbmA positively affect the uptake of proline-rich Bac7 peptides, suggesting a possible connection between their cellular functions.
INTRODUCTION

The yaiW gene is closely linked to the sbmA gene in the sequenced genomes of enteric bacteria (Fig. 1A) (1). These include *E. coli*, *Salmonella enterica* and other pathogenic species. In *E. coli* and *S. enterica* serovar Typhimurium, sbmA and yaiW could be shown to be part of the sigma E regulon, which is involved in the response to envelope stresses (2, 3). The close proximity of the sbmA and yaiW genes suggests that there could be a functional relationship between the SbmA and YaiW proteins, a possibility of particular interest because the molecular mechanism(s) of action of SbmA and its orthologs is still incompletely understood.

SbmA is an integral inner membrane protein whose function plays a positive role in the uptake of certain peptide substrates across the inner membrane (4, 5). These peptide substrates include microcins B17 and J25 (6, 7), bleomycin (8) and truncated, proline-rich Bac7 peptides (7, 9). Moreover, the *Sinorhizobium meliloti* and *Brucella abortus* orthologs of SbmA, BacA (10), are required for *S. meliloti* symbiosis (11) and *B. abortus* pathogenesis (12), while the *Mycobacterium tuberculosis* SbmA/BacA homolog, which also includes an ATPase domain (13), is involved in maintenance of *M. tuberculosis* chronic murine infections (14). Similarly to enteric SbmA, the *S. meliloti* and *B. abortus* BacA proteins mediate the uptake of bleomycin and truncated Bac7 proteins (15, 16). In addition, *S. meliloti* and *B. abortus* BacA deficient mutants have ~50% reduction in their LPS very-long chain fatty acids (VLCFA) content, demonstrating that BacA proteins play a key role in ensuring the complete modification of their LPS species with this unusual lipid (17, 18). In *S. meliloti*, lipid A VLCFA biosynthesis is dependent upon the **acpXL-lpxXL** cluster, which is absent in the genomes of enteric bacterial species (19). *S. meliloti* and *B. abortus* BacA-deficient mutants exhibit an increased detergent sensitivity relative to their parent strains, consistent with their altered LPS structures (18). In contrast, no detergent sensitivity phenotype was observed for *E. coli* mutants lacking SbmA (20), suggesting that they do not possess LPS alterations. BacA protein also protects *S. meliloti* against the toxic effects of a cysteine-rich nodule-specific (NCR) peptide produced during the legume symbiosis (21). NCR peptides have been shown to be essential
for bacterial differentiation into nitrogen fixing bacteroids (22). In addition, it was also suggested that the *Mycobacterium tuberculosis* BacA protein protects against human β-defensin 2 and that these human defensins might direct human pathogens towards a chronic infection state rather than leaving them in a potentially life threatening acute state (13). The molecular mechanism(s) underlying these complex effects of the SbmA/BacA family members remains unknown.

We therefore undertook an investigation of the hitherto uncharacterized *yaiW* gene with the long term goal of gaining insights into YaiW that would explain its function in enteric bacteria and might also inform our understanding of SbmA/BacA. In this paper, we report that *yaiW* is co-transcribed with *sbmA*, that its gene product YaiW is a palmitate-modified surface exposed outer membrane lipoprotein, and that both YaiW and SbmA positively affect the uptake of proline-rich Bac7 peptides, suggesting a possible connection between their cellular functions.
METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are described in Table S1 and S2, respectively. Unless stated otherwise, all *E. coli* strains were grown in either Lysogeny broth (LB) (23) prepared with 10 g l\(^{-1}\) NaCl or Müller-Hilton (MH) (24) medium or on LB or MH with 1.5 % (w/v) agar at 37°C. When required, for *E. coli* strains the antibiotics were added at the following concentrations: ampicillin (Ap, 100 µg ml\(^{-1}\)), kanamycin (Ka, 50 µg ml\(^{-1}\)) and chloramphenicol (Cp, 34 µg ml\(^{-1}\)). For all experiments, *S. meliloti* strains were grown in either LB prepared with 10 g l\(^{-1}\) NaCl or LB supplemented with 2.5 mM CaCl\(_2\) and 2.5 mM MgSO\(_4\) (LB/MC) for 48 hours at 30°C. When required, for *S. meliloti* strains the antibiotics were added at the following concentrations: streptomycin (Sm, 500 µg ml\(^{-1}\)), spectinomycin (Spc, 100 µg ml\(^{-1}\)) and tetracycline (Tc, 5 µg ml\(^{-1}\)).

Construction of mutants. A list of all plasmids and primers used can be found in Tables S2 and S3, respectively. The *sbmA::kan* and *yaiW::kan* deletions from the *E. coli* Keio collection (25) were transduced using bacteriophage P1 into MG1655. The kanamycin resistance cassettes were flipped out using pCP20 following a previously published method (25). The deletion mutants were confirmed by PCR using *Ecoli*sbmAprom_154 and *Ecoli*sbmA_R for Δ*sbmA*, *Ecoli*MG1655yaiW_F and *Ecoli*MG1655yaiW_R for Δ*yaiW* and *Ecoli*sbmAprom_154 and *Ecoli*MG1655yaiW_R primers for the Δ*sbmA*/Δ*yaiW* double mutant.

RNA isolation and RT-PCR. RNA from *S. Typhi* and *S. Typhimurium* was extracted from 25 ml of exponential phase cultures (OD\(_{600}\) = 0.4). Cultures were rapidly microcentrifuged, and pellets were frozen for 20 minutes in liquid nitrogen. Total RNA was extracted using the RNeasy minikit (Qiagen). Cells were lysed in RLT buffer (supplied with the kit) using Fast Protein tubes (Qbiogen) and the FastPrep FP120 cell disrupter (30 s at 6.5 m/s2). The RNA was then purified using the RNeasy minikit protocol. RT-PCR was performed using the SuperScript One-Step RT-PCR kit (Invitrogen) according to the manufacturer’s protocol. Co-transcription of *sbmA* and *yaiW* was analyzed using primers *sbmA_F*+930 und *yaiW_R*+74 (Table S2). To assess the quality of the
extracted RNA, the universal ribosomal RNA primers 8F and 1492R were also used. For all primer pairs, PCRs were also set up using the extracted RNA or S. Typhi or S. Typhimurium genomic DNA as a template using Taq DNA polymerase. RNA from E. coli HB101 was extracted from a bacterial cell sample of $10^9$ cells harvested at $OD_{600} = 0.4$ according to the User Manual of the Pure Link RNA Mini Kit (Invitrogen) and then treated with RNase-free DNase (Promega) according to the manufacturer’s instructions. cDNA was synthesized from approximately 1 µg of isolated RNA, using random hexamer primers (Promega). PCR based on the cDNA template (7 µl) was performed using $sbmA$-$yaiW$ fw and $sbmA$-$yaiW$ rev primers to amplify the intergenic region between $sbmA$ and $yaiW$. Specific primer pairs to amplify the end of $sbmA$ and the start of $yaiW$ were also used as a control (Table S2). As for the RNA extracted from S. Typhi or S. Typhimurium also for E. coli HB101 a positive control was set up with genomic DNA as a template using Taq DNA polymerase.

**Cloning of the yaiW genes.** The S. enterica serovar Typhimurium MC2 yaiW gene was amplified by PCR using $StmyaiW+$1F_NdeI and $StmyaiW$-1092R_XhoI. The yaiW gene was digested with NdeI and XhoI, ligated into plasmid pET22b and transformed into E. coli DH5α. The insert in pSTyaiW was then confirmed by sequencing and then the plasmid was transformed into the E. coli expression strains BL21(DE3) and BL21(DE3)pLysS. Expression from the T7lac promoter in pSTyaiW creates a C-terminal 6x His-tagged fusion protein. The E. coli yaiW gene was amplified by PCR using the $yaiW$-66_PstI and $yaiW$_rev_EcoRI primers. After digestion of the gene with PstI and EcoRI, $yaiW$ was ligated directly into plasmid pUT18 and transformed into E. coli DH5α. The insert was then confirmed by sequencing.

[^3H]-Palmitate labeling. To demonstrate that YaiW is a lipoprotein,[^3H]-palmitate labeling was performed following a modification of a previously published method (26). Stationary phase cultures of either BL21(DE3) with pET22b or pSTyaiW were diluted 10-fold into minimal medium [spizizen salts, 0.2% (w/v) glucose and 1 µg ml$^{-1}$] containing a protease inhibitor cocktail (Roche). The cultures were then grown until $OD_{600} = 0.6$ and then plasmid gene expression was induced by the addition of 1 mM IPTG for 45 minutes. E. coli host gene expression was then inhibited by the
addition of rifampicin (200 µg ml\(^{-1}\)) for 1 hour. To inhibit lipoprotein processing, cultures were then treated with and without globomycin (200 µg ml\(^{-1}\) dissolved in DMSO) for 20 minutes. The lipoproteins were then labeled by the addition of \(^{3}\text{H}\)-palmitate (50 µCi ml\(^{-1}\)) and incubated for a further 20 minutes. The cells were then pelleted in a microcentrifuge and washed 3 times with T-C buffer (10mM Tris-HCl pH 7.3 and 8 mM CaCl\(_2\)). The labeled lipoproteins were solubilized by boiling in protein loading buffer [2% (w/v) SDS, 10% (v/v) glycerol, 20 mM DTT, 0.02% (w/v) bromophenol blue, 63mM Tris-HCl pH 6.8] for 15 minutes and resolved by SDS-PAGE. After separation, the gels were fixed for 30 minutes in isopropanol:water:aceticacid (25:65:10) and then immersed in Amplify Solution (Amersham). The gel was dried and the radioactive bands detected by autoradiography.

**Membrane extraction and separation.** Overnight cultures of the defined strains were diluted into fresh LB and grown until an OD\(_{600}\) = 0.8. Plasmid gene expression was induced by the addition of 1 mM IPTG cultures and then the cells were incubated for a further 3 hours at 25°C. The cells were collected by centrifugation (32000 x g, 5 minutes) and re-suspended in 10 ml 25% sucrose (w/w) prepared in Tris Acetate (pH 7.8). One ml of lysozyme (2 mg ml\(^{-1}\)) was added on ice and after 2 minutes, 16 ml of 1.5 mM EDTA (pH 7.8) was added gradually over a time period of 6 minutes. Conversion of cells into spheroplasts was confirmed by microscopy. Cells were disrupted using a French Press (500 bar) and lysed cells were centrifuged at 9000 x g at 4°C for 15 minutes. The total membrane extraction in the supernatant was collected by ultracentrifugation at 100,000 x g at 4°C for 1 hour. The membrane pellet (~0.3 g) was homogenized in 2 ml of 25% sucrose (w/w) prepared in 10 mM Tris Acetate (pH 7.8).

The inner and outer membranes were separated at 4°C on a 30-60% (w/w) sucrose gradient prepared by layering from the bottom of a ultra-centrifuge tube (Beckman) 0.4 ml of 60%, 0.9 ml 55%, 2.2 ml of 50%, 45%, 40%, 1.3 ml of 35% and 0.4 ml of 30% (w/w) sucrose solutions prepared in 10 mM Tris Acetate and 0.5 mM EDTA (w/w) pH 7.8. The total membrane suspension was then layered onto the sucrose gradient and ultracentrifugation performed at 120,000 x g (SW41 rotor) at
4°C for 18 hours. Fractions (0.5 ml) were collected from the bottom of the tube and the OD$_{595}$ recorded and protein content determined by the Bradford assay. Ten µl aliquots of each fraction were mixed with 5 µl of Laemmli Buffer (27), denatured at 90°C for 10 minutes and then samples were resolved using a 10% (w/v) SDS-PAGE gel. The molecular weight of the proteins was determined using protein standards (Biorad). Separation of the membrane fractions was confirmed by using anti-OmpA and anti-SecA (28) antibodies, which will recognize proteins in the outer and inner membrane fractions, respectively.

**Western blots.** After being resolved by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Biorad) at 12 V for 1 hour and the membranes stained with Ponceau Red. Western blot analysis was performed according to the QIAexpress Detection and Assay Handbook (Qiagen). The rabbit anti-OmpA antibody and anti-SecA antibodies were used at titers of 1:80,000 and 1:10,000, respectively. A goat anti-rabbit secondary antibody conjugated to HRP (Imagenex) was used at a titer of 1:5,000. For detection of His-tagged proteins, the penta-His HRP conjugate (Qiagen) was used at a titer of 1:100,000. In all cases, the ECL plus kit (Amersham Bioscience) was used for the detection of HRP.

**Surface labeling of cells using NHS-LC-LC-Biotin.** Cell surface proteins of *E. coli* were labeled with NHS-LC-LC biotin as described previously (29). In brief, the desired strains were grown in LB with 100 µg ml$^{-1}$ Ap to an OD$_{600}$ ~ 0.8 and then expression of YaiW was induced by the supplementation of the growth medium with 1 mM IPTG. Then the cultures were continued to grow for 3 hours at 25°C, then washed 3 x in phosphate buffered saline (PBS) and finally resuspended to a final OD$_{600} = 10$ in PBS. Then NHS-LC-LC-Biotin (Pierce) was added to a final concentration of 2% and the reaction was stopped after 20 minutes by the addition of Tris (pH 7.5) to a final concentration of 250 mM. Whole cells lysates were produced using the French press at 20,000 PSI and then the protein concentration was determined using a Bio-Rad Bradford assay reagent (Bio-Rad).
**Bac7 sensitivity assay.** Bac7(1-16), Bac7(1-35) and the BODIPY fluorescently labeled derivative Bac7(1-35)-BY have been prepared as described in (30). To monitor bacterial growth inhibition, a suspension of 1x10^6 CFU ml^{-1} *E. coli* cells were grown in microtiter plates with periodic shaking at 37°C in the presence of 0.25 µM Bac7(1-16) or Bac7(1-35). The OD_{620} was measured every 10 minutes on a microtiter plate reader (Tecan Trading AG, Switzerland).

**Flow cytometry assays.** Uptake of BODIPY-labeled Bac7(1-35) in *E. coli* cells was determined by flow cytometry using a Cytomics FC500 instrument (Beckman-Coulter, Inc.) equipped as previously described (9). Cultures of mid-log phase bacteria were harvested, diluted to 10^6 CFU ml^{-1} in MH broth, incubated with 0.25 µM Bac7(1-35)-BODIPY® at 37°C for 10 minutes and analyzed immediately. All experiments were conducted in triplicate and data were expressed as Mean Fluorescence Intensity (MFI) ± S.D. Data analysis was performed with the FCS Express V3 software (De Novo Software, CA).

**Statistical analysis.** The significance of differences among bacterial strains was assessed using GraphPad Prism using ANOVA analysis followed by Bonferroni’s Multiple Comparison Test.

**Bioinformatics.** Bioinformatic analysis of *sbmA-yaiW* gene clusters in the different strains was performed using the Absynthe tool (http://archaea.u-psud.fr/absynte) using the full length *S. Typhimurium* LT2 *yaiW* gene sequence (Pubmed gene ID: 1251896) (31).
RESULTS

The *yaiW* gene is co-transcribed with *sbmA*. In the genomes of enteric bacteria the YaiW gene is located in close proximity to the *sbmA* gene, which encodes an inner membrane transport protein (Fig. 1A). To investigate whether the *sbmA* and *yaiW* genes are co-transcribed, RNA was extracted from the pathologically important bacterial strains *S. Typhimurium* MC2, and *S. Typhi* Ty2 and then RT-PCR was performed using forward and reverse primers internal to the *sbmA* and *yaiW* genes, respectively (Fig. 1B). In both cases, the RT-PCR reactions generated a product of ~0.6 kb (Fig. 1B, lanes II), which were sequenced and shown to be correct (data not shown). No PCR product was observed for the RNA preparations using the same primer pair in the absence of reverse transcriptase (Fig. 1B, lanes III). A 0.45 kb transcript of the two genes was also obtained in *E. coli* (Fig. 1C) confirming that the *sbmA* and *yaiW* genes are co-transcribed.

The *yaiW* gene encodes a lipoprotein. In the genomes of *S. Typhimurium* and *S. Typhi* the *yaiW* gene is annotated to encode a putative lipoprotein (NCBI reference No. NP_459372.1 and NP_806215.1, respectively) because it possesses a conserved lipobox sequence at its N-terminus (Fig. S1). To experimentally verify that YaiW is a lipoprotein, we constructed an *S. Typhimurium* MC2 Δ*yaiW::kan* mutant and then grew this mutant strain and its parent strain in the presence of [3H]-palmitate, which labels all lipoproteins (32). The [3H]-palmitate-labeled proteins were then resolved by SDS-PAGE. However, due to the large number of lipoproteins present in enteric bacteria, we were unable to detect any differences in the radioactive protein bands observed between the parent and Δ*yaiW* mutant using this approach (data not shown).

To overcome this technical difficulty, the *S. Typhimurium* MC2 *yaiW* gene was cloned into the *E. coli* expression vector, pET22B under control of the T7 lac promoter to create pST*yaiW*. Expression of *yaiW* from pET22B creates a C-terminal His-tagged protein, enabling detection of YaiW by Western blotting. To enable us to determine if YaiW is a lipoprotein, we grew *E. coli* BL21 (DE3) with either pST*yaiW* or the control vector, pET22B in the presence of IPTG to induce *yaiW* expression, and included [3H]-palmitate to radio-label lipoproteins. To prevent *E. coli* host
gene expression, the cultures were also treated with rifampicin, which inhibits *E. coli* RNA polymerases but not T7 RNA polymerase (33). Using this approach, we identified a radioactive band of ~39 kDa, corresponding to the mature YaiW C-terminal His-tagged protein, which was present in the extract from BL21 (DE3) with pSTyaiW but not with the control plasmid without insert (Fig. 2B). The antibiotic globomycin inhibits lipoprotein processing by interfering with the pro-lipoprotein signal peptidase (LspA), which results in the retention of the pro-lipoprotein in the inner membrane fused to its signal peptide (34, 35). We found that growth of *E. coli* BL21 (DE3) with pSTyaiW in the presence of globomycin inhibited the processing of the YaiW C-terminal His-tagged protein resulting in a larger, unprocessed YaiW protein (Fig. 2B). Therefore, since we determined that the *S. Typhimurium* YaiW protein is labeled with ^3^H-palmitate and the processing of this protein is inhibited by globomycin, our findings clearly demonstrate that YaiW is a lipoprotein.

**SbmA is not involved in the lipid modifications of YaiW or enteric LPS but complements the LPS lipid alteration of an *S. meliloti* BacA-deficient mutant.** Since *S. meliloti* and *B. abortus* BacA affect the VLCFA modification of lipid A, we examined whether its enteric homolog SbmA might be required for either the lipid modifications of YaiW or of enteric LPS. To investigate this, an sbmA::Tn5 insertion was transduced into *E. coli* BL23 (DE3) with pSTyaiW and then the ability of ^3^H-palmitate to label the YaiW-His tagged protein was investigated after IPTG induction. We found no reduction in the amount of ^3^H-palmitate labeling of YaiW and no difference in the mobility of the purified YaiW His-tagged protein by PAGE in the absence of the *E. coli* SbmA protein (Fig. 2C). In addition, we found no difference in the LPS species produced in the *E. coli sbmA* and *yaiW* mutants relative to the parent strain as determined by PAGE analysis (data not shown). Nor did we find any differences in the analyzed LPS fatty acid composition (C12:0, C14:0, 3-OH C14:0 and C16:0) of an *S. Typhimurium* sbmA mutant as determined by GC-MS (Fig. S2) following established procedures (17). In addition, we found no difference in the LPS species produced in the *E. coli sbmA* and *yaiW* mutants relative to the parent strain as determined by
PAGE analysis (data not shown). However, despite the fact that enteric bacterial species lack the LPS VLCFA biosynthesis cluster (19), we found that the plasmid-encoded *E. coli sbmA* gene (pAI351) was able to restore the LPS VLCFA content to the same extent as the plasmid-encoded *S. meliloti bacA* gene (pJG51A) in an *S. meliloti* BacA-deficient mutant in which the VLCFA biosynthesis is reduced (Table 1) (11).

**YaiW is an outer membrane lipoprotein.** In Gram-negative bacterial species, lipoproteins can be attached to either the inner or outer membrane (35). Inner membrane lipoproteins have an aspartic acid at residue 2 in the mature lipoprotein, which serves as an inner membrane retention sequence (35). In contrast, the *S. Typhimurium* YaiW protein has a serine residue in this position (Fig. 2A), suggesting that YaiW is an outer membrane lipoprotein (Pubmed gene ID: 1251896) (35). Creating an active antibody that targets YaiW proved to be very difficult and therefore we introduced the plasmid pSTyaiW, which encodes YaiW carrying a His tag, into *E. coli* to facilitate our investigation of the membrane localization of YaiW. Total membrane fractions from *E. coli* BL21 (DE3) with pSTyaiW grown in the presence of IPTG were isolated and the inner and outer membranes were then separated by sucrose-density gradient (30 – 60%) centrifugation (Fig. 3A). To ensure that effective separation of the inner and outer membranes had occurred, Western blots using antibodies against the outer membrane OmpA protein and the inner membrane SecA protein, were performed (36). We found the highest amount of the outer membrane protein OmpA in fraction 6 of the sucrose gradient (Fig. 3B) whereas the greatest amount of the inner membrane SecA protein was found in fractions 16 and 18 (Fig. 3C). Having demonstrated that we had achieved separation of the outer and inner membrane fractions, using a penta-His HRP conjugate we identified a band of the expected size for the mature YaiW-His tagged protein (39 kDa) in the membrane fractions obtained from *E. coli* BL23 (DE3) with pSTyaiW. This band was absent in the total membrane fraction prepared from BL21 (DE3) with the control plasmid, pET22B (labeled as negative control) (Fig. 3D). Since the same band was also observed in the lane containing purified YaiW-His tagged protein (labeled as positive control) (Fig. 3D), these findings show that the penta-
his HRP conjugate was specifically recognizing the YaiW C-terminal His-tagged protein in the membrane fractions. The highest amount of YaiW-His tagged protein was found in fraction 6 (Fig. 3D), which also contained the greatest amount of the outer membrane OmpA protein (Fig. 3B). Therefore, these findings demonstrate that \textit{yaiW} encodes an outer membrane lipoprotein.

\textbf{The YaiW lipoprotein is surface exposed.} A previous study described an effective way to detect surface exposed outer membrane proteins in \textit{E. coli} using the NHS-LC-LC-Biotin (Pierce) compound (Fig. 4A) (29). This compound forms stable bonds with primary amine groups (\(-NH_2\)) of amino acids (especially Lys) (Fig. 4A). Very detailed analysis demonstrated that NHS-LC-LC-Biotin labeled only surface exposed proteins and did not cross the \textit{E. coli} cell membrane to interact with any cytoplasmic or periplasmic proteins (29). Initially, we surface-labeled the surface proteins of the parent, the \textit{yaiW::kan} mutant and the \textit{ompA::kan} mutant strains from the single deletion mutant Keio strain collection with the NHS-LC-LC-Biotin compound (25). Streptavidin, which forms a very strong non-covalent bond with the vitamin biotin, was used to detect the protein-bound NHS-LC-LC-Biotin compound. The \textit{ompA::kan} mutant was used as a negative control for the Biotin labeling as it lacks the outer membrane protein OmpA (29). We found that a range of proteins of different sizes were surface exposed in all three strains. The outer membrane profiles of surface exposed proteins of the parent and \textit{yaiW::kan} mutant strains appeared to look very similar and no band that could be clearly attributed to YaiW (39 kDa) seemed to be missing for the \textit{yaiW::kan} mutant strain compared to the parent strain (Fig. 4B). However, OmpA (37.3 kDa), which is known to be surface exposed in \textit{E. coli}, is absent in the lane loaded with \textit{ompA::kan} mutant lysate (Fig. 4B) (29). Due to the presence of a large amount of surface proteins in the size range where YaiW (39 kDa) would be expected and the possibility that YaiW is only weakly expressed, \textit{S. Typhimurium} YaiW was over-expressed in \textit{E. coli} BL21(DE3) from pST\textit{yaiW} and purified after surface labeling. The YaiW proteins of \textit{E. coli} K12 and \textit{S. Typhimurium} LT2 are 92% similar (85% identical) (data not shown). In order to ensure that the His-tag itself was not recognized by Streptavidin-HRP, YaiW-His was purified after induction of its expression without prior surface
labeling by NHS-LC-LC-Biotin. A penta anti-His antibody (αHis) detected the purified YaiW-His protein (Fig. 4C) but the biotin specific Streptavidin-HRP did not (Fig. 4C). This finding proved that the His-tag does not interfere with Streptavidin-HRP and that Streptavidin-HRP detection was exclusive to biotin-labeled proteins. Over-expression of YaiW (39 kDa) in an E. coli BL21(DE3) parent strain and purification of His-tagged proteins subsequent to surface labeling resulted in a clean band detected by a penta anti-His antibody (Fig. 4D). No band was observed with pET22b only. The same was found when YaiW was expressed from pSTyaiW in a ΔsbmA mutant (Fig. 4D). Streptavidin-HRP detected biotin labeled proteins of the same size for the parent and the ΔsbmA mutant with pSTyaiW (Fig. 4E). No bands corresponding to the size of YaiW were observed for the parent strain with pET22b only (Fig. 4D and E). We therefore demonstrated that YaiW is surface exposed in both E. coli and S. Typhimurium.

**YaiW is involved in the uptake of proline-rich Bac7 peptides.** The finding that sbmA and yaiW are co-transcribed suggests that they might possibly be involved in a related process in enteric bacteria. It was shown previously that mutations in sbmA decrease the susceptibility of enteric bacterial species towards different types of antimicrobial peptides (AMPs) such as bleomycin, microcin B17 and truncated Bac7 peptides (6, 9, 20). Therefore, to investigate whether YaiW is involved in the sensitization of E. coli towards AMPs, we compared the sensitivity of isogenic deletion mutants of sbmA and yaiW towards two fragments of Bac7 that differed in length. In the presence of sub-lethal concentrations of both Bac7(1-35) (data not shown) and Bac7(1-16) the deletion of yaiW conferred a moderate increase in the ability of the strain to grow (Fig. 5A). To investigate whether the increased viability of the ΔyaiW mutant in the presence of the peptide could be due to a decreased internalization of the peptide, we measured the uptake of fluorescently labeled Bac7(1-35)-BY in both the ΔsbmA and ΔyaiW mutants. We found that, similarly to the effect of deleting sbmA, deletion of yaiW resulted in a decrease of almost 50% of peptide internalization (Fig. 5B). In addition the sensitivity of isogenic deletion mutants of sbmA, yaiW and sbmA/yaiW towards bleomycin sulfate and microcin B17 was also tested. We also found that deletion of sbmA
conferred decreased sensitivity of *E. coli* towards these two AMPs but deletion of *yaiW* had no effect. The Δ*sbmA*/Δ*yaiW* double mutant had the same sensitivity to bleomycin and microcin B17 as the Δ*sbmA* single mutant (data not shown).

In order to investigate whether the YaiW and SbmA proteins might interact with each other we expressed *yaiW* and *sbmA* fused to the two subunits of the adenylate cyclase of *Bordetella pertussis*. Since this two-hybrid system detects only interactions occurring at the cytoplasm or inner membrane level (37), a truncated form of YaiW in which the lipobox signature was removed was used. No detectable interaction between SbmA and YaiW was revealed in these experimental conditions, suggesting either that the two proteins may not be associated at the inner membrane level or that interaction of SbmA and YaiW could occur in the periplasm, which would be out of the context of this two hybrid system (data not shown).
DISCUSSION

In this study, we demonstrate that enteric yaiW is in an operon with sbmA and that YaiW is a surface-exposed lipoprotein. To our knowledge only a few lipoproteins have been proven to be surface exposed in E. coli. These include Wza(K30), a lipoprotein required for surface polysaccharide polymerisation (38), TraT, a lipoprotein involved in F-sex factor conjugation (39) and CsgG, a lipoprotein important for the assembly of curli fibers (40, 41). Recently, the most abundant lipoprotein in E. coli, Lpp has also been demonstrated to be exposed at the cells surface using the NHS-LC-LC-Biotin compound we employed in our study (29).

We also show that, like SbmA, YaiW, affects the internalization of the proline-rich peptide Bac7(1-35). Until now, such functional relationship between both proteins could only be suggested based on their location near each other in bacterial genomes. A plausible explanation for this relationship is that YaiW contributes positively to the proline-rich peptide crossing the outer membrane, while SbmA contributes positively to it crossing the cytoplasmic membrane. Although a direct interaction of the SbmA and YaiW proteins was not observed in a bacterial two-hybrid system (data not shown), which detects interactions in the cytoplasm, it may still be possible that both proteins indirectly interact in the periplasm via a periplasmic protein. Additionally, there appears to be some specificity to the functional relationship of SbmA and YaiW since we did not observe any effects of YaiW on the sensitivity to microcin B17 or bleomycin. Further studies will be required to clarify the physiological role of this relationship. Finally, in the course of our study, we made the interesting observation that, although VLCFA’s are not present in enteric bacteria and SbmA does not affect the lipid modifications of E. coli LPS, when introduced into S. meliloti SbmA can substitute for BacA’s role in the VLCFA modification of S. meliloti LPS.
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References


Table 1. SbmA complements the lipid A alteration of an *S. meliloti* BacA-deficient null mutant

<table>
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<tr>
<th>Fatty acid</th>
<th>Amount in mol/2mol GlcN</th>
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<tr>
<td></td>
<td>pAI351</td>
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<tr>
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<tr>
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Figure 1. The *sbmA* gene is co-transcribed with *yaiW*. (A) Schematic representation of the *sbmA*-*yaiW* gene regions in the genomes of selected Gram negative bacteria. Genomic data was imported and analyzed by the Absynthe tool (http://archaea.u-psud.fr/absynte). (B) Agarose gel analysis of DNA products generated by RT-PCR using either primers internal to the 16S rRNA gene (*rrsH*) (lanes I) or a forward primer internal to *sbmA* and a reverse internal to *yaiW* (lane II). The expected products of 1.5 and 0.6 kb for lanes I and II were obtained, respectively. A negative control was also performed using the same primers as in lane 1 but only Taq polymerase was added in the absence of reverse transcriptase (lane III). M=Invitrogen 1kb ladder. (C) Agarose gel analysis of DNA products generated by RT-PCR from *E. coli* HB101 using either primers specific for the tail of *sbmA* (lane 1) or for the start of *yaiW* (lane 2) or a forward primer internal to *sbmA* and a reverse internal to *yaiW* (lane 3). The expected products of 0.32, 0.12 and 0.45 kb for lanes 1,
2 and 3 were obtained, respectively. A negative and positive control were also performed using the same primers as in each lane but adding no template or gDNA respectively.

**Figure 2. YaiW is a lipoprotein.** (A) The N-terminus sequence of the *S. enterica* serovar Typhimurium YaiW protein is shown indicating the lipobox and predicted second residue in the mature protein. (B) *E. coli* BL21 (DE3) with either the control vector (pET-22B) or pSTaiW (as indicated) were grown in the presence of 1mM IPTG, 200 µg ml⁻¹ rifampicin and [3H]-palmitate and the lipoproteins present detected as described in the materials and methods. To inhibit lipoprotein processing, cultures were also treated with 200 µg ml⁻¹ globomycin as defined. The predicted molecular masses of the full length and truncated YaiW his tagged protein are 41.2 and 39.2 kDa, respectively. (C) Cultures of either BL21 (DE3) or BL21 (DE3) *sbmA::Tn5* with either pT22B or pSTaiW (as defined) were grown and the lipoproteins labeled as described in Figure 2B. Experiments were only performed in the absence of globomycin.
Figure 3. The YaiW-his tagged protein is localized to the outer membrane. Cultures of E. coli BL21 (DE3) with pSTyaiW were grown in the presence of 1 mM IPTG. The total membranes were then isolated, the inner and outer membranes separated and resolved by sucrose-gradient density centrifugation. (A) The turbidity (solid line) and protein concentration (dashed line) of the different membrane fractions obtained from the sucrose density gradient centrifugation. (B-D) Western blots using 10 µl aliquots of the different membrane fractions were then performed using antibodies against either the known outer membrane OmpA protein (B), the known inner membrane SecA protein (C) or the His tag (D). The total membrane fraction (10 µl) from BL21 (DE3) with the control plasmid (-YaiW) and the purified YaiW-his tagged protein (3 µl; YaiW-his) were also included in (D) as negative and positive controls, respectively.
Figure 4. A plasmid encoded His-tagged *Salmonella* YaiW protein is surface exposed in *E. coli*. (A) Chemical structure of NHS-LC-LC-Biotin. (B) The defined Keio collection parent and mutant strains were treated with NHS-LC-LC-Biotin. After cell lysis 10 µg of whole cell lysate was separated using SDS-PAGE and probed using Streptavidin-HRP. (C) *E. coli* BL21(DE3) harbouring pSTyaiW was grown with 1mM IPTG. After cell lysis the whole cell lysate was run through a Ni-NTA agarose bead loaded gravity column and the elution fractions were separated using SDS-PAGE and probed using a penta anti-his antibody or Streptavidin-HRP. (D and E) The defined strains were grown in the presence of 1 mM IPTG and then treated with NHS-LC-LC-Biotin. After cell lysis the whole cell lysates were run through a Ni-NTA agarose bead loaded gravity column and the elution fractions were separated using SDS-PAGE and probed using a penta anti-his antibody (D) or Streptavidin-HRP (E). All data shown are representative of at least two independent experiments.
Figure 5. *E. coli* YaiW is important for peptide stress resistance. (A) Growth kinetics of *E. coli* BW25113, ΔsbmA and ΔyaiW strains in MH Broth in absence (upper panel) or in presence of 0.25 µM Bac7(1-16). Bacterial suspensions of $1 \times 10^6$ cells/ml have been grown for 4 hours and the OD at 620 nm has been measured every 10 minutes. Results are the mean of three independent experiments. (B) Uptake of Bac7(1-35)-BY in *E. coli* BW25113, ΔsbmA and ΔyaiW strains measured as medium fluorescence intensity (M.F.I.). Results are the mean of more than three independent experiments. The significant values *** $p \leq 0.0004$ and ** $p \leq 0.0015$ were determined using ANOVA followed by Bonferroni’s post test. All datasets represent trends observed in at least two independent experiments.