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JOURNAL OF BACTERIOLOGY, Feb. 2007, p. 1244–1253 Vol. 189, No. 4
0021-9193/07/$08.00+0 doi:10.1128/JB.00773-06

C-Terminal WxL Domain Mediates Cell Wall Binding in *Enterococcus faecalis* and Other Gram-Positive Bacteria▽

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Received 29 May 2006/Accepted 30 August 2006

Analysis of the genome sequence of *Enterococcus faecalis* clinical isolate V583 revealed novel genes encoding surface proteins. Twenty-seven of these proteins, annotated as having unknown functions, possess a putative N-terminal signal peptide and a conserved C-terminal region characterized by a novel conserved domain designated WxL. Proteins having similar characteristics were also detected in other low-G+C-content gram-positive bacteria. We hypothesized that the WxL region might be a determinant of bacterial cell location. This hypothesis was tested by generating protein fusions between the C-terminal regions of two WxL proteins in *E. faecalis* and a nuclease reporter protein. We demonstrated that the C-terminal regions of both proteins conferred a cell surface localization to the reporter fusions in *E. faecalis*. This localization was eliminated by introducing specific deletions into the domains. Interestingly, exogenously added protein fusions displayed binding to whole cells of various gram-positive bacteria. We also showed that the peptidoglycan was a binding ligand for WxL domain attachment to the cell surface and that neither proteins nor carbohydrates were necessary for binding. Based on our findings, we propose that the WxL region is a novel cell wall binding domain in *E. faecalis* and other gram-positive bacteria.

Gram-positive bacteria contain a variety of extracellular proteins, some of which are crucial for adaptation and survival in the environment. These proteins participate in various important functions, such as nutrient catabolism, adhesion for niche colonization, translocation, and defense against the host immune system in the case of invading pathogens (35). At this point, cell surface-exposed proteins secreted via the Sec pathway can be classified into the following seven categories according to their means of associating with the cell surface (1, 4, 6, 8): (i) membrane-spanning proteins; (ii) lipoproteins (52, 55); (iii) cell wall-anchored proteins, which are covalently linked to the peptidoglycan at the carboxyl terminus via the LPXTG motif (34, 48); (iv) proteins containing LysM domains that bind to peptidoglycan, most of which are cell wall hydrolases (23, 44, 51); (v) GW proteins, which interact with lipoteichoic acids on the bacterial cell surface and also with mammalian cells (as shown for InlB and Ami of *Listeria monocytogenes* [2, 21, 22, 33]); (vi) choline-binding proteins, which bind to phosphorylcholine in the cell wall (12); and (vii) proteins containing surface layer homology domains, which interact with peptidoglycan or cell wall-associated polymers, forming a crystalline surface layer (41). The latter class is restricted to bacilli and lactobacilli so far (32). A new C-terminal domain designated WxL was recently predicted based on in silico global genome analyses of *Lactobacillus plantarum* (25), *Lactobacillus coryniformis* (47), and *Lactobacillus sakei* (5). In these studies, neither biological properties nor cell localization was examined.

With the ever-increasing availability of bacterial genome sequences, the major protein families described above have been confirmed to be present in numerous gram-positive bacteria, including *Enterococcus faecalis* (38). *E. faecalis* is a natural inhabitant of the gastrointestinal and vaginal tracts and the oral cavity of humans. It is also a constituent of the natural microflora of a variety of fermented food products. However, *E. faecalis* is also an opportunistic pathogen that can infect patients with severe underlying diseases, and it is a major cause of acquired hospital infections worldwide. The large range of intrinsic or acquired antibiotic resistances in *E. faecalis* strains contributes to the difficulty of treating infections. A dozen virulence factors have been reported so far (for reviews, see references 24 and 53). However, little is known about the mechanism of *E. faecalis* pathogenesis. Information derived from genome sequence analysis of the *E. faecalis* clinical isolate V583 suggested that additional surface proteins might be involved in *E. faecalis* pathogenesis and/or adaptation to the environment (38).

In this study, we identified novel WxL proteins in *E. faecalis* and other low-G+C-content gram-positive bacteria. Members of this family of proteins are characterized by a conserved C-terminal WxL domain. Using fusions to a reporter protein, we demonstrated that this C-terminal region is involved in determining bacterial cell surface localization and may interact with the peptidoglycan.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Enterococci were grown in M17 broth or agar supplemented with 0.5% glucose at 37°C without aeration. *Escherichia coli* and *Bacillus subtilis* strains were grown aerobically in Luria-Bertani broth or agar at 37°C. *Listeria innocua* and *Staphylococcus aureus* strains were grown aerobically at 37°C in brain heart infusion medium. *Streptococcus agalactiae* and *Lactobacillus johnsonii* were cultivated anaerobically at 37°C in M17 medium supplemented with 0.2% glucose and in MRS medium, respectively. Antibiotics were used at the following concentrations: erythromycin, 10 μg/ml for *E. faecalis* and 150 μg/ml for *E. coli*; and ampicillin, 80 μg/ml.

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▽ Published ahead of print on 8 September 2006.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
E. coli TG1	*supE hsdD5 thi* (Δ*lac-proAB*) F′ (*traD36 proAB-lacZΔM15*)	15
E. faecalis JH2-2	Fus^r Rif^r; plasmid-free wild-type strain	20
B. subtilis 168	*trpC2*	3
S. agalactiae NEM316	Serotype III isolated from neonatal blood culture	16
L. innocua ATCC 33090	Wild type	43
L. johnsonii ATCC 33200	Wild type	13
S. aureus RN4220	Wild type	26
Plasmids		
pGEM-T	Amp^r, linearized with 3′ T overhangs, ori ColE1	Promega
pG+host9	Erm^r, oriTS	31
pVE3618	Amp^r, ori ColE1; P_{usp45}::sp_{Usp45}::*nucB*	28
pVE5524	Amp^r Erm^r, ori ColE1 pAMb1; P_{59}::sp_{Usp45}::*nucA*::cwa_{M6}	9
pUC18	Amp^r, ori ColE1	58
pIL253	Erm^r, ori pAMβ1	50
pVE14055	Amp^r, pUC18 with P_{usp45}::sp_{Usp45}::*nucB*::$CWA_{M6(277-415)}$	This study
pVE14059	Amp^r, pGEM-T with 1,478 bp including first 105 bp and last 93 bp of *ef2686*	This study
pVE14060	Amp^r, pUC18 with P_{usp45}::sp_{Usp45}::*nucB*	This study
pVE14065	Amp^r Erm^r, pG+host9/pVE14059	This study
pVE14068	Amp^r Erm^r, pUC18/pIL253 with P_{usp45}::sp_{Usp45}::*nucB*	This study
pVE14069	Amp^r Erm^r, pUC18/pIL253 with P_{usp45}::sp_{Usp45}::*nucB*::$CWA_{M6(277-415)}$	This study
pVE14074	Erm^r, pG+host9 with first 105 bp and last 93 bp of *ef2686*	This study
pVE14089	Amp^r, pGEM-T with last 224 amino acids of EF0392 ($EF0392_{WxL2}$)	This study
pVE14091	Amp^r, pGEM-T with P_{usp45}::*nucB*	This study
pVE14093	Amp^r, pUC18 with P_{usp45}::sp_{Usp45}::*nucB*::$EF0392_{WxL2}$	This study
pVE14096	Amp^r Erm^r, pUC18/pIL253 with P_{usp45}::sp_{Usp45}::*nucB*::$EF0392_{WxL2}$	This study
pVE14098	Amp^r Erm^r, pUC18/pIL253 with P_{usp45}::*nucB*	This study
pVE14101	Amp^r, pGEM-T with last 126 amino acids of EF2686 ($EF2686_{WxL2}$)	This study
pVE14105	Amp^r, pUC18 with P_{usp45}::sp_{Usp45}::*nucB*::$EF2686_{WxL2}$	This study
pVE14109	Amp^r Erm^r, pUC18/pIL253 with P_{usp45}::sp_{Usp45}::*nucB*::$EF2686_{WxL2}$	This study
pVE14114	Amp^r, pGEM-T with $EF2686_{597-723}$ with last 22 amino acids deleted ($EF2686_{WxL1}$)	This study
pVE14115	Amp^r, pGEM-T with $EF2686_{597-723}$ with last 98 amino acids deleted ($EF2686_{WxL0}$)	This study
pVE14116	Amp^r, pGEM-T with $EF0392_{541-764}$ with last 17 amino acids deleted ($EF0392_{WxL1}$)	This study
pVE14117	Amp^r, pUC18 with $EF0392_{541-764}$ with last 118 amino acids deleted ($EF0392_{WxL0}$)	This study
pVE14118	Amp^r, pUC18 with P_{usp45}::sp_{Usp45}::*nucB*::$EF2686_{WxL1}$	This study
pVE14119	Amp^r, pUC18 with P_{usp45}::sp_{Usp45}::*nucB*::$EF2686_{WxL0}$	This study
pVE14120	Amp^r, pUC18 with P_{usp45}::sp_{Usp45}::*nucB*::$EF0392_{WxL1}$	This study
pVE14121	Amp^r, pUC18 with P_{usp45}::sp_{Usp45}::*nucB*::$EF0392_{WxL0}$	This study
pVE14122	Amp^r, erm^r, pUC18/pIL253 with P_{usp45}::sp_{Usp45}::*nucB*::$EF2686_{WxL1}$	This study
pVE14123	Amp^r, erm^r, pUC18/pIL253 with P_{usp45}::sp_{Usp45}::*nucB*::$EF2686_{WxL0}$	This study
pVE14124	Amp^r, erm^r, pUC18/pIL253 with P_{usp45}::sp_{Usp45}::*nucB*::$EF0392_{WxL1}$	This study
pVE14125	Amp^r, erm^r, pUC18/pIL253 with P_{usp45}::sp_{Usp45}::*nucB*::$EF0392_{WxL0}$	This study

[a] ori ColE1 and ori pAMβ1 refer to the replicon.

General DNA techniques. General molecular biology techniques were performed by standard methods (46). Restriction enzymes, T4 DNA ligase, and the Klenow fragment were used according to manufacturer's instructions. PCR amplification was performed using either a Perkin-Elmer (Boston, MA) or Eppendorf (Hamburg, Germany) apparatus. When necessary, PCR products and DNA restriction fragments were purified with QIAquick kits (QIAGEN, Valencia, CA). Plasmids were purified using QIAprep Miniprep or Midiprep (QIAGEN). Electrotransformation of *E. coli* and *E. faecalis* was carried out as described previously (10, 11), using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA). Plasmid inserts were sequenced with Amersham dye terminator chemistry using a MegaBACE DNA sequencing machine (Amersham Biosciences, Freiburg, Germany).

Plasmid construction. (i) Construction of fusions to the *S. aureus nuc* reporter gene. The *nuc* open reading frame comprises a long signal sequence followed by a region encoding a propeptide and the mature nuclease. An EcoRI/HindIII fragment from pVE3618 (Table 1) contained the first 440 bp of *nuc* (encoding the N-terminal end of the nuclease proprotein, NucB) fused in frame with the *Lactococcus lactis* Usp45 signal peptide (sp_{Usp45}) and expressed under control of the P_{usp45} promoter. A HindIII/BamHI fragment from pVE5524 (Table 1) contained the last 145 bp of *nuc* fused to the sequence encoding the M6 cell wall-anchored region of *S. pyogenes* [$cwa_{M6(277-415)}$]. Both EcoRI/HindIII and HindIII/BamHI fragments were ligated between the BamHI and EcoRI sites of pUC18 to construct the P_{usp45}::sp_{Usp45}::*nuc*::cwa_{M6} fusion. The resulting pVE14055 plasmid was digested with EcoRV/NarI to eliminate the cwa_{M6} region, treated with the Klenow polymerase, and self-ligated to obtain pVE14060, which contained the P_{usp45}::sp_{Usp45}::*nuc* cassette in pUC18.

EF2686 and EF0392 are *E. faecalis* putative proteins predicted from the V583 genome (38). A DNA fragment encoding the last 126 residues of the *E. faecalis* EF2686 open reading frame ($EF2686_{598-723}$), designated $EF2686_{WxL2}$, was PCR amplified from JH2-2 chromosomal DNA with primers 5′-GACGGATCCCCTATTTTGCAC-3′ and 5′CCGCTCGAGCTGCAAATCGCTAGTAG-3′ containing BamHI and XhoI restriction sites (underlined), respectively, and was cloned into pGEM-T to obtain pVE14101. A second fragment encoding the last 224 residues of the *E. faecalis* the EF0392 open reading frame ($EF0392_{541-764}$), designated $EF0392_{WxL2}$, was PCR amplified from JH2-2 chromosomal DNA with primers 5′-GTCTCTCGAGGTTGGCATTAC-3′ and 5′-GACGGATCCTTATGGGCCTG-3′ containing BamHI and XhoI restriction sites (underlined), respectively, and was cloned into pGEM-T to obtain pVE14089. The nucleotide sequences of the inserts were validated by sequencing.

The BamHI/XhoI fragment of pVE14055 was replaced by the BamHI/XhoI fragment of pVE14101 corresponding to $EF2686_{WxL2}$ and by the fragment of pVE14089 corresponding to $EF0392_{WxL2}$ to obtain pVE14105 and pVE14093, respectively. *E. coli-E. faecalis* shuttle plasmids were obtained by fusion between pIL253 and pVE14055, pVE14060, pVE14093, and pVE14105 at the EcoRI site

to obtain pVE14069, pVE14068, pVE14096, and pVE14109, respectively. In these plasmids the erythromycin resistance gene (*ermB*) and *nuc* fusions were transcribed in the same orientation.

A plasmid expressing cytoplasmic Nuc was constructed from pVE14068 by deletion of sp_{Usp45}. A 347-bp fragment that included P_{usp45} and the start codon and a 555-bp fragment corresponding to the region encoding the Nuc proprotein were PCR amplified from pVE14068 using primers 5′-**CGCGGATCC**GATTACATGGATTGGATTAG-3′ containing a BamHI restriction site (underlined) and 5′-**CAT**AACTGTTCTTTTTTAATTTTTC-3′ (start codon indicated by bold type) and primers 5′-**ACGCGTCGAC**TCAGGCGATCTCGAGATCGA-3′ containing a SalI restriction site (underlined) and 5′-GAAAAATTAAAAAAGAACAGTTATGTATGCATCACAAACAGATAACG-3′, respectively. These two PCR products were fused by PCR performed with the external primers and cloned into pGEM-T. The resulting plasmid, pVE14091, was fused at the SalI/BamHI sites of pIL253 to obtain pVE14098.

(ii) Construction of Nuc::EF2686 and Nuc::EF0392 fusions with WxL deleted. The C-terminal (Ct) regions of EF2686 lacking the last C-terminal 22 residues ($EF2686_{598\text{-}701}$), designated $EF2686_{WxL1}$, and the last 98 residues ($EF2686_{598\text{-}625}$), designated $EF2686_{WxL0}$, were respectively recovered by PCR using the chromosomal *ef2686* gene of JH2-2 with oligonucleotides 5′-CGGGATCCCTAGGCCGTCCCTCCAGG-3′ and 5′-CCGCTCGAGCTGCAAATCGCTAGTAG-3′ containing BamHI and XhoI restriction sites (underlined), respectively, and oligonucleotides 5′-CGGGATCCCTACCCTGTTTGACGAGTATC-3′ and 5′-CCGCTCGAGCTGCAAATCG CTAGTAG-3′ containing BamHI and XhoI restriction sites (underlined), respectively. The corresponding PCR products were cloned into pGEM-T to obtain pVE14114 and pVE14115, respectively. The Ct regions of EF0392 lacking the last C-terminal 17 residues ($EF0392_{541\text{-}747}$), designated $EF0392_{WxL1}$, or the last 118 residues ($EF0392_{541\text{-}646}$), designated $EF0392_{WxL0}$, were respectively obtained by PCR using the *ef0392* gene in the chromosome of JH2-2 with oligonucleotides 5′-CGGGATCCCTATCTACCTTGATTTGCTG-3′ and 5′-GTCTCTCGAGGTTGGCATTAC-3′ containing BamHI and XhoI restriction sites (underlined), respectively, and oligonucleotides 5′-CGGGATCCCTAACGTAAATATGGATTTTC-3′ and 5′-GTCTCTCGAGTATATTGCTGG-3′ containing BamHI and XhoI restriction sites (underlined), respectively. The PCR products were cloned into pGEM-T to obtain pVE14116 and pVE14117, respectively. The BamHI/XhoI fragment of pVE14055 was replaced by the BamHI/XhoI fragments of pVE14114, pVE14115, pVE14116, and pVE14117 to obtain pVE14118, pVE14119, pVE14120, and pVE14121, respectively. The nucleotide sequences of cloned PCR products were systematically confirmed by sequencing. Cointegrates were obtained by constructing fusions between pIL253 and pVE14118, pVE14119, pVE14120, and pVE14121 at the EcoRI site, which yielded pVE14122, pVE14123, pVE14124, and pVE14125, respectively.

Construction of an in-frame *ef2686* deletion mutant of strain JH2-2. A markerless Δ*ef2686* deletion mutant of *E. faecalis* was constructed by cloning two DNA fragments corresponding to the chromosomal DNA regions upstream and downstream of *ef2686* in plasmid pGhost9 (31). A DNA fragment encompassing the last 747 bp at the 3′ end of the *ef2687* gene and the first 105 bp of the *ef2686* gene was amplified by PCR from JH2-2 chromosomal DNA with primers 5′-TATTCGATGTTGGCGTTGG-3′ and 5′-GGAGGATGCGATTGTTTCG-3′. A second fragment encompassing the last 112 bp of the *ef2686* gene, 533 bp of the *ef2685* gene, and the 5′ end the *ef2684* gene was PCR amplified from JH2-2 chromosomal DNA with primers 5′-CGAAACAATCGCATCCTCCTTATCTGTTCCTGGAGGGAC-3′ and 5′-TATCTGGATTCACTGGATCG-3′. The two PCR products were fused by PCR using the external primers, and the resulting product was cloned into pGEM-T, yielding pVE14059. This plasmid was then fused to the pG+host9 plasmid at the PstI site. The resulting plasmid, pVE14065, was digested with PvuII to eliminate pGEM-T, yielding plasmid pVE14074, which was then electroporated into JH2-2. Transformants were selected at the permissive temperature (28°C) on M17 plates with erythromycin. *ef2686* mutants were constructed essentially as described previously (42). Briefly, overnight cultures grown under the same conditions were diluted and shifted to the nonpermissive temperature (42°C) in the presence of erythromycin to select single-crossover integrants. Plasmid excision by a second recombination event was stimulated by growing integrants at 28°C without erythromycin. Overnight cultures were plated at 42°C without erythromycin to select an *ef2686* double-crossover mutation. Successful targeted mutation of *ef2686* in strain VE14258 was first identified by PCR screening and was confirmed by Southern blot analysis.

Cell fractionation. *E. faecalis* cell fractionation was performed using previously described methods (36, 39). Bacterial proteins were separated into protoplast (P), cell wall (CW), and supernatant (SN) fractions. Five milliliters of an exponential-phase culture (A_{600}, ~0.6) was pelleted. The supernatant was filtered with a 0.22-μm filter, and the proteins were precipitated on ice with 12% trichloroacetic acid (TCA), centrifuged, rinsed with acetone, and resuspended in deionized water with protease inhibitor cocktail (Roche Diagnostics, Meylan, France). The bacterial pellet was washed once in cold TES (10 mM Tris-HCl [pH 8], 10 mM EDTA [pH 8], 17% sucrose), resuspended in 120 μl of TES containing lysozyme (5 mg/ml) and protease inhibitor cocktail, and incubated for 15 min at 4°C and for 15 min at 37°C. To maintain protoplast integrity, $MgCl_2$ was added at a final concentration of 50 mM prior to centrifugation at 9,000 × *g* for 10 min at 4°C. Proteins in the cell wall fraction were recovered by TCA precipitation of the resulting supernatant and resuspension in 150 μl of deionized water containing protease inhibitor cocktail with 0.05 N NaOH. Protoplasts were resuspended in 120 μl of deionized water containing protease inhibitor cocktail and were disrupted by four freeze-thaw cycles, followed by addition of 1% sodium dodecyl sulfate (SDS) and heating for 3 min at 95°C. Fifty microliters of 4× loading buffer (40) was added to each protein sample to obtain a final volume of 200 μl, and samples were heated for 5 min at 95°C. To ensure that the cell wall fraction was devoid of cytoplasmic contamination, the glucose-6-phosphate dehydrogenase activity was determined for protoplasts and cell wall fraction aliquots by determining the A_{340} after addition of 0.2 mM NADP and 0.6 mM glucose-6-phosphate, as described previously (14).

Binding of Nuc fusion proteins to bacterial cells or peptidoglycan. To study the binding of Nuc fusion proteins added exogenously to bacteria, 5 ml of an exponential-phase bacterial culture was collected by centrifugation and washed with cold phosphate-buffered saline (PBS) (0.02 M NaH_2PO_4, 0.08 M Na_2HPO_4, 1.5 M NaCl). The cell pellet or 150 μg of insoluble peptidoglycan from *B. subtilis* (Sigma-Aldrich, Lyon, France) that was briefly sonicated using a Sonicator Vibracell (model 75042; Fisher Bioblock Scientific, Illkirch, France) was gently resuspended in an initial volume of filter-sterilized culture supernatants of strains expressing fusion proteins. The mixture was incubated for 30 min at 37°C and centrifuged for 5 min at 9,000 × *g*. The proteins remaining in the supernatant were precipitated on ice with 12% TCA. The cell pellet was washed with cold PBS, resuspended in a PBS solution containing lysozyme (5 mg/ml), and incubated for 15 min at 4°C and for 15 min at 37°C. Whole-cell proteins were resuspended as described above and heated for 5 min at 95°C, and nuclease production was analyzed by Western blotting.

Immunoblots. Nuclease detection by Western blotting was performed as previously described (27). Briefly, 0.1 volume of protein extract prepared as described above was heated for 5 min at 95°C and electrophoresed on an SDS–12% polyacrylamide gel. A prestained molecular marker was systematically included (Euromedex, Mundolsheim, France). Proteins were transferred from gels to polyvinylidene difluoride membranes (Millipore, Guyancourt, France) by electroblotting. Nuc protein was detected with monoclonal antibody 7F11-B7 (Bionor, Skien, Norway) diluted 1:5,000. Bound primary antibodies were detected with horseradish peroxidase-coupled anti-mouse antibodies diluted 1:5,000 (Roche, Meylan, France). Antibody binding was detected by enhanced chemiluminescence with an ECL kit (Amersham Biosciences).

Cell treatments. Proteins and carbohydrates were eliminated from *E. faecalis* cell wall fractions and *E. faecalis* cells by methods used previously for other gram-positive bacteria (51, 54). Briefly, cell wall fractions were treated with 10% SDS or 10% TCA for 15 min at 100°C, followed by four washes with cold PBS. For exogenous binding of Nuc fusions with the cell wall, CW fractions of strains expressing Nuc fusions were precipitated on ice with 12% TCA and then subjected to various treatments, including 0.5% SDS at 37°C for 30 min, 0.1% Triton X-100 and 0.1% Tween 20 at room temperature for 30 min, and 150 mM NaCl, 10% acetone, and 5% methanol at 4°C for 15 min. The cell walls were centrifuged and washed twice with cold PBS, and proteins were prepared as described above. Whole cells of *E. faecalis* JH2-2 and VE14258 (~5×10^8 cells/ml) were treated with (i) either 250 μg/ml or 1 mg/ml of trypsin (Sigma) in 1 ml of 0.1 M Tris-HCl (pH 8.0)–0.01 M $CaCl_2$ for 1 h at 37°C, followed by addition of 125 μg/ml or 1.25 mg/ml of soybean trypsin inhibitor (Sigma) and incubation at room temperature for 40 min, or (ii) 0.01 M or 0.05 M sodium periodate (Sigma) prepared in 0.01 M PBS in the dark at 4°C for 20 min. Cells were washed four times with cold PBS before binding experiments were performed with filter-sterilized culture supernatants of strains expressing fusion proteins.

Immunofluorescence microscopy. Immunofluorescence staining was performed with whole cells of an exponential-phase culture after fixation with 4% paraformaldehyde, followed by treatment with 1 mg/ml lysozyme for 20 min at 37°C. Treated cells were washed with PBS and incubated with monoclonal antibody 7F11-B7 (Bionor) diluted 1:50 in PBS–0.5% bovine serum albumin overnight at room temperature. The cells were washed three times with PBS, and bound antibodies were detected by incubation with Alexa 555-coupled anti-mouse antibodies (Sigma) diluted 1:200 in PBS–0.5% bovine serum albumin for 1 h at room temperature. After this, cells were washed three times with PBS and resuspended in 100 μl of PBS. A 15-μl sample was applied to a glass slide, air

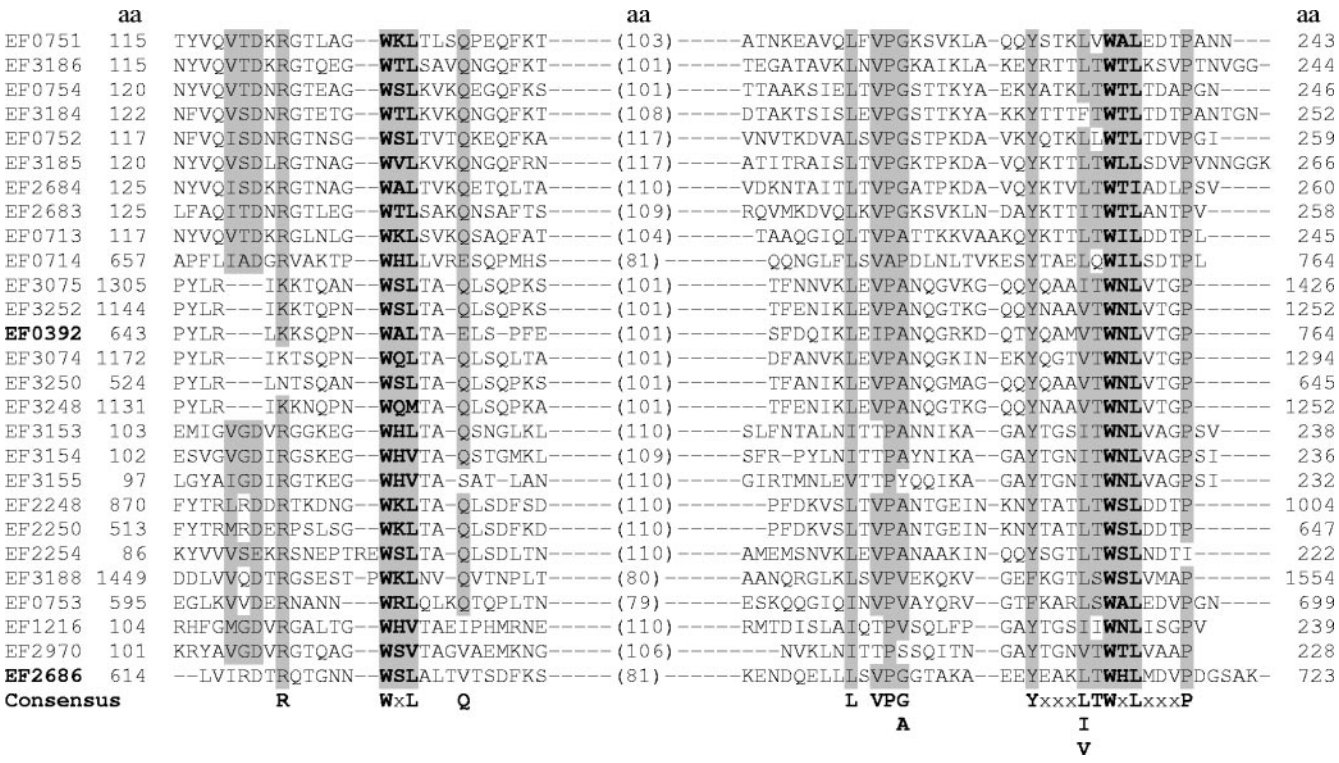

FIG. 1. Multiple-amino-acid sequence alignment of the 27 proteins of *E. faecalis* strain V583 using the ClustalW program. WxL domains are indicated by bold type and shading. The distances between the two WxL domains are indicated in parentheses. Amino acids that are identical or similar are indicated by shading. The groups of amino acids considered similar are I, L, M, and V; A, S, G, and T; H, K, and R; D, E, N, and Q; and F, W, and Y.

dried, and heat fixed. Analysis was performed with a confocal microscope (Olympus Fluoview IX70).

Bioinformatics analyses. A pan-genomic search for potential exported proteins was performed for the *E. faecalis* clinical isolate V583 sequence (http://www.tigr.org/) using both Signal P 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and Psort (http://psort.nibb.ac.jp/form.html), with selection for putative signal peptides of gram-positive bacteria. Transmembrane helices were searched for with TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and DAS server (http://www.sbc.su.se/~miklos/DAS/). Protein alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/). To identify similarities with and distribution of WxL proteins in other bacterial species, BLAST analyses of protein sequences were performed at the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Identification of a new family of exported proteins. Approximately 300 proteins having a signal peptide were predicted based on bioinformatics analyses of *E. faecalis* V583, using both Signal P 3.0 and Psort. A total of 227 of these proteins were also predicted to be anchored membrane proteins with at least one transmembrane domain or a lipid-anchored residue. Of the remaining putative exported proteins, 38 contained an LPXTG cell wall-anchoring motif or a LysM domain, whereas 63 had none of the previously described surface localization motifs. Interestingly, amino acid alignments of 27 of the latter proteins (ranging from 222 to 1,554 amino acids long), generated with ClustalW, revealed several conserved residues in the last 130 C-terminal residues along with a highly conserved YXXX(L/I/V)TWXLXXXP terminal motif and a second WxL proximal motif (Fig. 1). Proteins containing these motifs are referred to here as WxL proteins. The distance between the two WxL motifs varied from 79 to 117 residues. All these proteins had unknown functions. Interestingly, EF2686 and EF2250 belong to the well-described internalin protein family (7, 45). However, the TIGR annotation (http://cmr.tigr.org/tigr-scripts/CMR/shared/GenePage.cgi?locus=EF2686) indicates that EF2686 is a pseudoprotein. Among the genes encoding *E. faecalis* WxL proteins, *ef1216* and *ef0392* were orphan genes, whereas the 25 other genes were organized in eight clusters containing pairs of paralogous genes encoding WxL proteins and, in five cases, a conserved hypothetical membrane protein.

To investigate how widespread the WxL proteins are among bacterial proteins, extended BLAST analyses were performed using the amino acid sequences of the C-terminal region of EF2686 to search for related proteins in bacterial genomes available at the NCBI. A total of 65 additional potential exported proteins having C-terminal conserved residues and a WxL domain followed by a well-conserved YXXX(L/I/V)TW XLXXXP motif were found in the genomes of 10 low-G+C-content gram-positive bacteria (Table 2). The two WxL domains were separated by between 66 and 247 residues. Similar cluster organizations, including the conserved hypothetical membrane protein open reading frame, were observed for the majority of proteins belonging to the WxL family (Table 2).

We propose that these proteins are members of a new family of secreted proteins having unknown functions characterized by a well-conserved YXXX(L/I/V)TWXLXXXP motif and a WxL motif upstream at the C-terminal end.

TABLE 2. Distribution of WxL proteins in the bacterial genome

Bacterial genome	No. of:	
	WxL proteins	WxL clusters
Enterococcus faecalis V583	27	8
Lactobacillus plantarum	19[a]	7
Lactobacillus sakei	15[b]	5[b]
Lactococcus lactis	7	1
Listeria innocua	6	3
Listeria monocytogenes	4	2
Enterococcus faecium DO	4	1
Bacillus cereus	3	1
Lactobacillus casei	5	2
Lactobacillus coryniformis	1[c]	
Pediococcus pentosaceus	1	

[a] Data from reference 25.
[b] Data from reference 5.
[c] Data from reference 47.

Proteins fused to the C-terminal regions of EF2686 and EF0392 are located at the cell surface. We investigated the role of the C-terminal regions of two WxL family proteins in protein location. The internalin-like protein EF2686 is encoded by a gene in a cluster of genes encoding two other WxL proteins and a transmembrane protein (see above). Protein EF0392 is encoded by a putative monocistronic gene having an unknown function and has close paralogues in the WxL family. The last 126 and 224 C-terminal residues of the EF2686 and EF0392 WxL proteins, respectively, were fused to the staphylococcal nuclease (Nuc) reporter. The resulting Nuc fusions contained the complete WxL domain (WxL2). It has been demonstrated previously that heterologous signals can be added at the N and C termini of Nuc to target it to specific locations in various lactic acid bacteria (9, 39).

We first determined whether Nuc could be expressed and exported to various compartments of *E. faecalis*. For this purpose, we used strain JH2-2 expressing the P_{usp45}::sp_{Usp45}::*nuc* construct, which produces a secreted Nuc protein. Protein extracts were prepared from the protoplast (P), cell wall (CW), and supernatant (SN) fractions, and the Nuc location was analyzed by Western blotting, using Nuc-specific monoclonal antibodies. The P and CW fractions had no cytoplasmic contamination, as determined by the cytosolic enzyme glucose-6-phosphate dehydrogenase activity (data not shown). Western blot analysis using Nuc-specific antibodies revealed two bands that were detected exclusively in the SN fraction, and these bands corresponded to NucB proprotein and the mature NucA form (Fig. 2A). This result demonstrates that Nuc can be efficiently produced and secreted in *E. faecalis* using *L. lactis usp45* transcription, translation, and secretion signals.

The P_{usp45}::sp_{Usp45}::*nuc* cassette was fused in frame at its 3′ end to (i) the C-terminal region of the streptococcal M6 protein containing the LPXTG cell wall anchor (CWA) domain [P_{usp45}::sp_{Usp45}::*nuc*::$cwa_{M6(277-415)}$], (ii) the C-terminal region of WxL protein EF2686 (P_{usp45}::sp_{Usp45}::*nuc*::$ef2686_{WxL2}$), and (iii) the C-terminal region of WxL protein EF0392 (P_{usp45}::sp_{Usp45}::*nuc*::$ef0392_{WxL2}$). For the strain expressing Nuc-CWA_{M6}, three bands were detected in the P fraction, and their sizes were the sizes expected for the precursor and mature forms of anchored NucB-CWA_{M6} and covalently anchored NucB-CWA_{M6} or NucA-CWA_{M6} (Fig. 2B). The two latter forms were also detected in the CW and SN fractions, indicating that the cell wall anchoring via CWA_{M6} is partial. The strains expressing fusions to the WxL regions (Nuc-$EF2686_{WxL2}$ and Nuc-$EF0392_{WxL2}$ [36 and 46 kDa, respectively]) generated patterns similar to those observed for the CWA_{M6} fusions (Fig. 2C and D). These results suggest that the last 127 and 224 residues of the EF2686 and EF0392 WxL proteins, respectively, are involved in Nuc association with the bacterial cell surface. To obtain insight into the physical nature of interactions between the Nuc fusions and the bacterial surface, the CW fractions of strains expressing Nuc-$EF2686_{WxL2}$ and Nuc-$EF0392_{WxL2}$ were treated with 0.5% SDS, 0.1% Triton X-100, 0.1%, Tween 20, 150 mM NaCl, 10% acetone, or 5% methanol. The CW fraction proteins were then analyzed by Western blotting. Only the SDS treatment disrupted binding of Nuc-$EF2686_{WxL2}$ and Nuc-$EF0392_{WxL2}$ to the cell wall fraction (data not shown). This result strongly indicates that the fusion proteins are likely to have strong, noncovalent interactions with the bacterial cell wall or surface. However, we cannot rule out the possibility that the presence of these fusion proteins in the P fraction reflected inefficient protein export resulting from saturation of the secretion machinery due to a high level of protein expression.

Nuc-$EF2686_{WxL2}$ and Nuc-$EF0392_{WxL2}$ added exogenously bind to *E. faecalis* whole cells. To distinguish between binding ability and inefficient protein translocation, we performed exogenous binding experiments using *E. faecalis* whole cells as the target. VE14258 and JH2-2 cells were incubated with culture supernatants of *E. faecalis* strains expressing the secreted Nuc protein, Nuc-$EF2686_{WxL2}$, or Nuc-$EF0392_{WxL2}$. After washing to eliminate nonspecific binding, proteins of whole cells and supernatants were precipitated and analyzed by Western blotting (Fig. 3). Nuc was recovered exclusively in the supernatant fraction. In contrast, both Nuc-$EF2686_{WxL2}$ and Nuc-$EF0392_{WxL2}$ were detected in the cell fractions, indicating that the WxL regions of EF2686 and EF0392 allow the Nuc fusion proteins to bind to *E. faecalis*. These results suggest that WxL regions promote protein interactions with the enterococcal surface.

WxL domains are involved in cell surface localization. We investigated whether the two C-terminal WxL domains present in

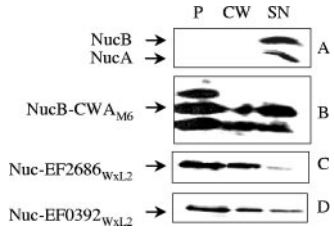

FIG. 2. Localization of Nuc fusions by fractionation. Proteins from *E. faecalis* strains expressing P_{usp45}::sp_{Usp45}::*nucB* (A), P_{usp45}::sp_{Usp45}::*nucB*::$cwa_{M6(277-415)}$ (B), P_{usp45}::sp_{Usp45}::*nucB*::$EF2686_{WxL2}$ (C), and P_{usp45}::sp_{Usp45}::*nucB*::$EF0392_{WxL2}$ (D) were fractionated in three compartments, protoplasts (P), cell walls (CW), and the supernatant (SN). Equivalent amounts of proteins were subjected to SDS–12% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, and immunodetection was performed using Nuc monoclonal antibodies. The arrows indicate bands migrating at the expected positions for the proteins indicated. The lower bands in panels A and B migrated at the position of the protein from which the N-terminal propeptide was also cleaved (26). The upper band in panel B migrated at the position of the full-length protein containing the signal peptide (38).

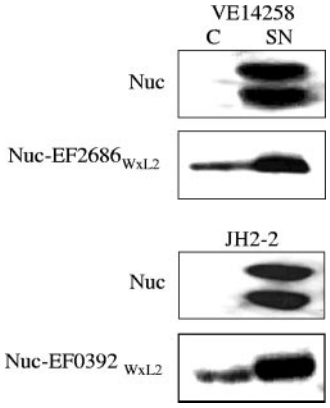

FIG. 3. Interaction of Nuc fusions with JH2-2 and VE14258 whole cells. Whole cells of strain VE14258 were incubated with supernatants of strains expressing secreted Nuc and Nuc-EF2686$_{WxL2}$. Strain JH2-2 was incubated with supernatants of strains expressing secreted Nuc and Nuc-EF0392$_{WxL2}$. Proteins were fractionated in two compartments, cells (C) and the supernatant (SN), and were analyzed by Western blotting.

all the WxL proteins contributed to cell surface association. We constructed C-terminally truncated Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ fusions (Fig. 4A). The locations of fusions lacking one WxL domain (Nuc-EF2686$_{WxL1}$ or Nuc-EF0392$_{WxL1}$) or both WxL domains (Nuc-EF2686$_{WxL0}$ or Nuc-EF0392$_{WxL0}$) in JH2-2 were analyzed. Nuc-EF2686$_{WxL1,}$ from which the distal WxL domain was deleted, was barely detected in the CW fraction, indicating that the terminal WxL region is important for binding to the bacterial surface. When both WxL domains were deleted, Nuc-EF2686$_{WxL0}$ was detected exclusively in the SN fraction. This suggests that besides having an impaired association with the cell wall fraction, this fusion protein had increased protein export efficiency (Fig. 4B). Similar but less marked results were obtained when experiments were performed with the Nuc-EF0392$_{WxL2}$ fusion (Fig. 4B); deletion of the distal WxL domain slightly impaired detection in the cell wall fraction, whereas deletion of both WxL domains nearly eliminated the fusion protein in the cell wall. These results suggest that the WxL domains of both EF2686$_{WxL2}$ and EF0392$_{WxL2}$ are important for the cell surface location. In all cases, decreased detection in the cell wall fraction was correlated with increased detection in the SN fraction.

When culture supernatants of JH2-2 strains expressing one of the fusions with WxL deleted were incubated with JH2-2 and VE14258 cells, they did not interact with bacterial cells (Fig. 4C). All these results strongly suggest that the WxL domains of EF2686 and EF0392 play a major role in the cell surface location of the Nuc fusion proteins.

Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ are located at the cell surface of *E. faecalis*. We used immunofluorescence with Nuc monoclonal antibodies to confirm that Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ fusions are located on the bacterial surface, as suggested by the Western blot results. Using standard protocols (57) with the strains expressing the Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ fusion proteins or the anchored control protein, Nuc-CWA$_{M6}$, we did not detect the protein at the *E. faecalis* cell

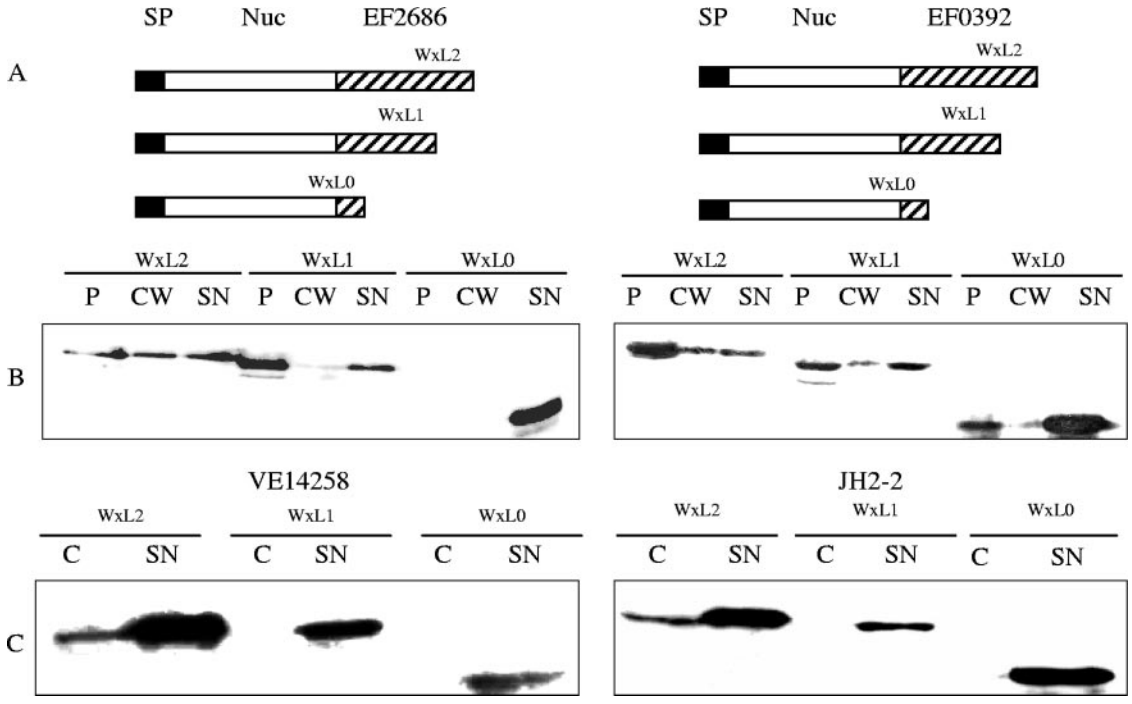

FIG. 4. (A) Schematic diagrams of Nuc fusion proteins and derivatives used in this study. SP, signal peptide. The open bars represent Nuc; the cross-hatched bars represent the C-terminal domains of EF2686 and EF0392. (B) Western blot analysis of protein extracts of P, CW, and SN fractions from *E. faecalis* JH2-2 strains expressing Nuc fused to proteins with complete or deleted WxL regions, as indicated above the lanes. (C) Reassociation of Nuc fusions with *E. faecalis* cells. VE14258 cells were incubated with culture supernatants of strains expressing Nuc-EF2686$_{WxL2}$, Nuc-EF2686$_{WxL1}$, and Nuc-EF2686$_{WxL0}$. Strain JH2-2 cells were incubated with culture supernatants of strains expressing Nuc-EF0392$_{WxL2}$, EF0392$_{WxL1}$, and EF0392$_{WxL0}$. Cells (C) and supernatants (SN) were separated and analyzed by Western blotting. Immunoblotting was done using Nuc monoclonal antibodies.

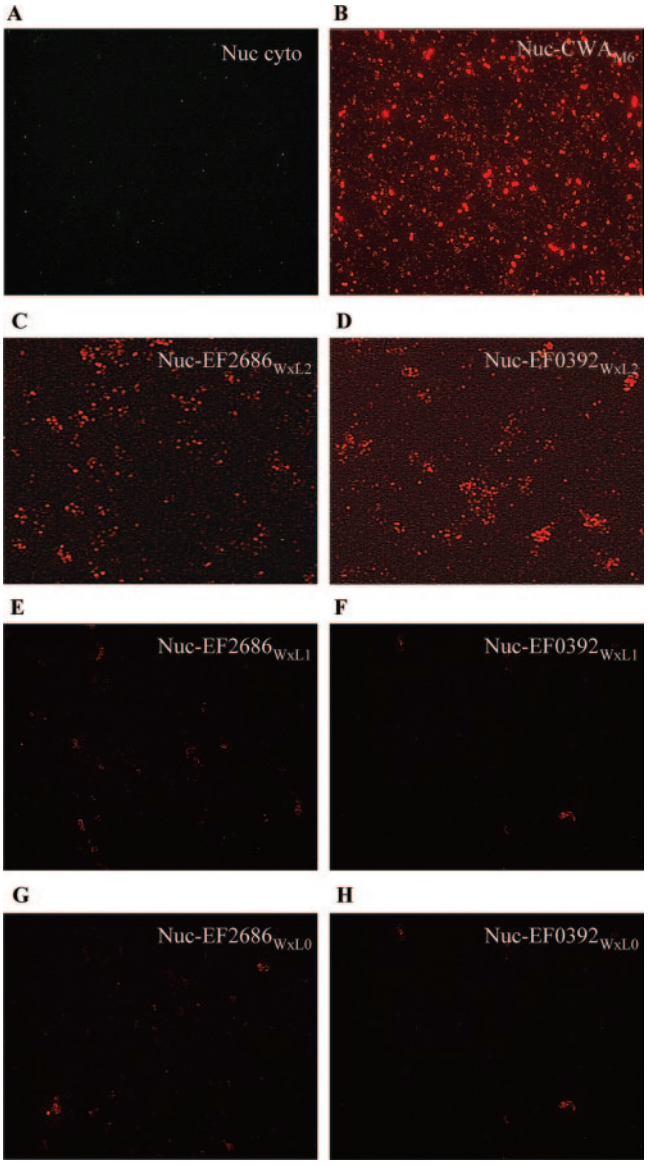

FIG. 5. Detection of Nuc fusion proteins on the *E. faecalis* cell surface by immunofluorescence. Strains expressing the different fusions were treated with lysozyme, labeled with anti-Nuc monoclonal antibodies and Alexa 555 secondary antibodies, and visualized by fluorescence.

surface, suggesting that proteins may be poorly accessible for Nuc antibody recognition. Therefore, we partially digested the peptidoglycan by gentle lysozyme treatment (see Materials and Methods) of *E. faecalis* strains before immunostaining. No protein was detected on control strains expressing cytoplasmic Nuc (Fig. 5A), validating the conclusion that the bacterial cell integrity was maintained. In these conditions, Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ were readily detected on the *E. faecalis* cell surface (Fig. 5C and D), as was Nuc-CWA$_{M6}$ (Fig. 5B), suggesting that the cell surface ligand must be buried in the peptidoglycan or that peptidoglycan accessibility was hindered by cell wall polysaccharides. No protein was detected on cells expressing the fusions with one or both WxL domains deleted, confirming that the two domains are implicated in surface association (Fig. 5E to H). These data further confirmed that EF2686$_{WxL2}$ and EF0392$_{WxL2}$ are implicated in *E. faecalis* cell surface association.

WxL domain of EF2686 and EF0392 confers binding to peptidoglycan. We determined whether Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ could bind in *trans* to other gram-positive bacteria. Since Nuc reportedly has affinity for cell surfaces of several bacteria (9, 30), we first confirmed that Nuc itself did not bind to *B. subtilis*, *S. agalactiae*, *S. aureus*, *L. innocua*, or *L. johnsonii* (data not shown). Prepared cells of these strains were then incubated with filtered supernatants of JH2-2 strains expressing Nuc-EF2686$_{WxL2}$, Nuc-EF0392$_{WxL2}$, or their WxL deletion derivatives. Western blot analysis of whole-cell proteins indicated that Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ were present in cell fractions of all the strains tested, whereas Nuc fusions with the two WxL domains deleted showed no cell association (Fig. 6). We concluded that the C-terminal region of EF2686 and EF0392 allows binding of Nuc fusions to gram-positive bacteria, possibly suggesting that there is a common cell surface component.

Interactions between the WxL domain of EF2686 and EF0392 and the bacterial surface were examined in *trans*. We incubated supernatant fractions of *E. faecalis* strains expressing Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ fusions with cell wall fractions of JH2-2 and VE14258 that were pretreated with SDS or TCA or with JH2-2 and VE14258 whole cells that were pretreated with trypsin and sodium periodate to strip the surface of proteins and carbohydrates, respectively (see Materials and Methods). Cell walls and cell proteins were analyzed by Western blotting using anti-Nuc monoclonal antibodies for detection. Both Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ bound to the stripped cell wall or cell fraction, indicating that the binding component was not removed by any of the treatments (data not shown). These results indicated that neither proteins nor carbohydrates were needed for binding and suggested that the cell wall ligand for WxL proteins could be the peptidoglycan.

Since we had evidence that Nuc-WxL fusions bound to *B. subtilis*, we examined binding to commercially available purified peptidoglycan from *B. subtilis*. Insoluble peptidoglycan was incubated with supernatants of *E. faecalis* strains expressing secreted Nuc, Nuc-EF2686$_{WxL2}$, and Nuc-EF0392$_{WxL2}$ and their derivatives with WxL deleted. Pellet and supernatant fractions were analyzed by Western blotting. Whereas secreted Nuc was found only in the supernatant fraction, Nuc-EF2686$_{WxL2}$ and Nuc-EF0392$_{WxL2}$ coprecipitated with insoluble peptidoglycan. These results indicate that peptidoglycan is likely to be a ligand for binding of WxL proteins to the bacterial cell surface. Nuc-EF2686$_{WxL1}$ lacking one WxL domain was weakly detected in the peptidoglycan, and Nuc-EF2686$_{WxL0}$ lacking both WxL domains was not detected. These results indicate that WxL domains are needed for binding to peptidoglycan. Much less pronounced results were obtained for Nuc-EF0392$_{WxL2}$ derivatives, possibly suggesting that the 106 residues located upstream of the WxL region in this construct may influence the efficiency of binding to peptidoglycan. These results suggest that the peptidoglycan is a binding ligand for WxL domain attachment to the cell surface.

DISCUSSION

In this work, we identified a novel family of *E. faecalis* putative exported proteins characterized by a C-terminal WxL

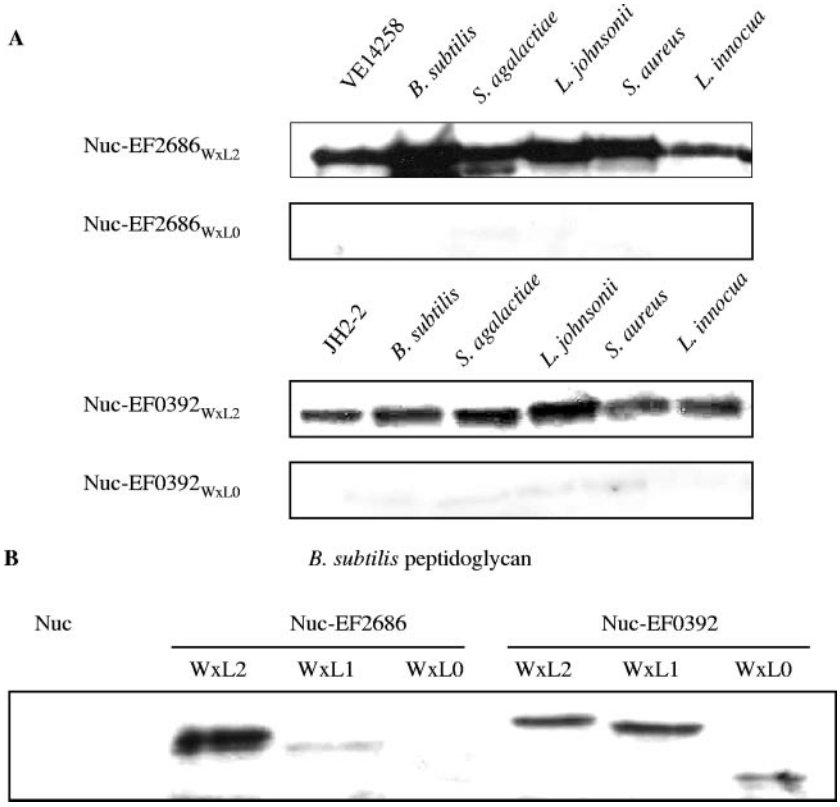

FIG. 6. Interaction of Nuc fusion proteins with gram-positive bacterial cells (A) and peptidoglycan of *B. subtilis* (B). (A) Strain VE14258 and *B. subtilis*, *S. agalactiae*, *L. johnsonii*, *S. aureus*, and *L. innocua* strains were incubated with supernatants of cultures of strains expressing Nuc-EF2686$_{WxL2}$ and Nuc-EF2686$_{WxL0}$ or expressing Nuc-EF0392$_{WxL2}$ and Nuc-EF0392$_{WxL0}$. Cell proteins were analyzed by Western blotting, using anti-Nuc antibodies. (B) Interaction of Nuc fusions proteins. One hundred fifty micrograms of insoluble peptidoglycan of *B. subtilis* was incubated with supernatants of strains expressing Nuc-EF2686$_{WxL2}$, Nuc-EF2686$_{WxL1}$ and Nuc-EF2686$_{WxL0}$, or Nuc-EF0392$_{WxL2}$, Nuc-EF0392$_{WxL1}$, and Nuc-EF0392$_{WxL0}$. Suspensions were then centrifuged, and the pellets were subjected to SDS-polyacrylamide gel electrophoresis analysis and Western blot analysis using anti-Nuc monoclonal antibody.

domain, which we showed is involved in bacterial cell surface association. WxL is characterized by conserved residues, and one highly conserved YXXX(L/I/V)TWXLXXXP motif and a second WxL motif are systematically present.

We demonstrated that (i) C-terminal regions of *E. faecalis* EF2686 and EF0392 confer protein localization to the *E. faecalis* cell wall, (ii) WxL domains are necessary for noncovalent interactions of the exported Nuc fusion proteins with the cell wall, and (iii) the WxL region interacts with the peptidoglycan. We found that WxL proteins appear to be localized both at the cell surface and in the culture medium, despite the fact that cell wall association involves strong noncovalent interactions. To explain this, we speculated that the presence of WxL proteins in the supernatant probably reflects peptidoglycan turnover. Together, these results provide the first biological evidence that the C-terminal WxL region is a cell surface localization domain.

Nuc fusion proteins were shown to be surface exposed when immunofluorescence microscopy was used. However, the results suggest that the WxL proteins might be buried within the cell wall. Poor antibody accessibility to surface-exposed proteins has been reported previously. When expressed in *E. faecalis*, *Streptococcus mutans* antigen P1 (SpaP) was identified as molecule located in the cell wall fraction by immunoblotting, whereas it was barely detected by immunofluorescence (19). Recent studies showed that streptococcal capsule or major surface proteins were involved in the camouflage of several surface proteins and could reduce either antibody recognition (for Blr of *S. agalactiae* and Slr of *S. pyogenes* [56]) or cell adhesion capacity (for cell wall-anchored proteins in *S. pneumoniae* [17, 37]). Our results with *E. faecalis* may similarly indicate that cell wall polysaccharides, which include lipoteichoic acid, teichoic acid, a rhamnopolysaccharide, and a variable capsular polysaccharide (18), might hinder detection of and/or access to surface-exposed proteins.

The presence of proteins with two WxL domains in only a subset of gram-positive bacteria belonging to the low-G+C-content group suggests that WxL proteins may be related to a common ecological niche. Like *E. faecalis*, lactobacilli, *Listeria*, and *B. cereus* occur in various habitats, such as the gastrointestinal tracts of humans and animals, as well as fermented foods. Binding of WxL proteins to various gram-positive bacteria suggests that they might mediate interactions between bacteria. For example, Cpf, a WxL protein of *L. coryniformis*, mediated coaggregation between *E. coli* and *Campylobacter* (47). However, no evidence suggested that the WxL domain was involved in aggregation activity. Interestingly, we found that several genes encoding WxL proteins identified in *E. fae-*

calis V583 (NCBI accession number AE016830) are not present in various isolates (29). We speculated that WxL proteins may facilitate bacterial adaptation to specific biotopes. During the review process, Siezen et al. described in silico identification of clusters of proteins with WxL domains in various low-G+C-content gram-positive bacteria, including *E. faecalis* (49). These recent bioinformatic analyses, which suggest that N-terminal domains of certain WxL proteins exhibit structural similarity with concanavalin A-like lectins/glucanases are in keeping with our hypothesis. Siezen et al. speculated that these proteins might participate in binding and/or degradation of complex saccharides found in plants (49).

In conclusion, in this study we identified a novel cell surface protein family characterized by a WxL localization domain that mediates noncovalent binding to the cell surface of various bacteria. While this work clearly showed that WxL domains are involved in binding to peptidoglycan, we have not ruled out possible interactions of these proteins with other surface components. Identification of the peptidoglycan moiety that interacts with the WxL domain and functional characterization of members of the WxL protein family remain to be investigated.

ACKNOWLEDGMENTS

We thank P. Langella and J. C. Piard for providing plasmids pVE3618 and pVE5524, respectively. We are grateful to A. Gruss, I. Poquet, and M. P. Chapot-Chartier for their critical reviews of the manuscript.

This work was supported by the Institut National de la Recherche Agronomique. S.B. was supported by fellowships from the Région Ile-de-France and the Fondation de la Recherche Médicale.

REFERENCES

1. **Bergmann, S., and S. Hammerschmidt.** 2006. Versatility of pneumococcal surface proteins. Microbiology **152:**295–303.
2. **Braun, L., S. Dramsi, P. Dehoux, H. Bierne, G. Lindahl, and P. Cossart.** 1997. InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. Mol. Microbiol. **25:**285–294.
3. **Burkholder, P. R., and N. H. J. Giles.** 1947. Induced biochemical mutations in *Bacillus subtilis*. Am. J. Bot. **34:**345–348.
4. **Cabanes, D., P. Dehoux, O. Dussurget, L. Frangeul, and P. Cossart.** 2002. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends Microbiol. **10:**238–245.
5. **Chaillou, S., M. C. Champomier-Verges, M. Cornet, A. M. Crutz-Le Coq, A. M. Dudez, V. Martin, S. Beaufils, E. Darbon-Rongere, R. Bossy, V. Loux, and M. Zagorec.** 2005. The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. Nat. Biotechnol. **23:**1527–1533.
6. **Chhatwal, G. S.** 2002. Anchorless adhesins and invasins of gram-positive bacteria: a new class of virulence factors. Trends Microbiol. **10:**205–208.
7. **Cossart, P.** 2002. Molecular and cellular basis of the infection by *Listeria monocytogenes*: an overview. Int. J. Med. Microbiol. **291:**401–409.
8. **Desvaux, M., E. Dumas, I. Chafsey, and M. Hebraud.** 2006. Protein cell surface display in gram-positive bacteria: from single protein to macromolecular protein structure. FEMS Microbiol. Lett. **256:**1–15.
9. **Dieye, Y., S. Usai, F. Clier, A. Gruss, and J. C. Piard.** 2001. Design of a protein-targeting system for lactic acid bacteria. J. Bacteriol. **183:**4157–4166.
10. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
11. **Dunny, G. M., L. N. Lee, and D. J. LeBlanc.** 1991. Improved electroporation and cloning vector system for gram-positive bacteria. Appl. Environ. Microbiol. **57:**1194–1201.
12. **Fernandez-Tornero, C., R. Lopez, E. Garcia, G. Gimenez-Gallego, and A. Romero.** 2001. A novel solenoid fold in the cell wall anchoring domain of the pneumococcal virulence factor LytA. Nat. Struct. Mol. Biol. **8:**1020–1024.
13. **Fujisawa, T., Y. Benno, T. Yaeshima, and T. Mitsuoka.** 1992. Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson et al. 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981). Int. J. Syst. Bacteriol. **42:**487–491.
14. **Fujita, Y., R. Ramaley, and E. Freese.** 1977. Location and properties of glucose dehydrogenase in sporulating cells and spores of *Bacillus subtilis*. J. Bacteriol. **132:**282–293.
15. **Gibson, T. J.** 1984. Studies on the Epstein-Barr virus genome. Ph.D. thesis. University of Cambridge, Cambridge, United Kingdom.
16. **Glaser, P., C. Rusniok, C. Buchrieser, F. Chevalier, L. Frangeul, T. Msadek, M. Zouine, E. Couve, L. Lalioui, C. Poyart, P. Trieu-Cuot, and F. Kunst.** 2002. Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. Mol. Microbiol. **45:**1499–1513.
17. **Gor, D. O., X. Ding, D. E. Briles, M. R. Jacobs, and N. S. Greenspan.** 2005. Relationship between surface accessibility for PpmA, PsaA, and PspA and antibody-mediated immunity to systemic infection by *Streptococcus pneumoniae*. Infect. Immun. **73:**1304–1312.
18. **Hancock, L. E., and M. S. Gilmore.** 2002. The capsular polysaccharide of *Enterococcus faecalis* and its relationship to other polysaccharides in the cell wall. Proc. Natl. Acad. Sci. USA **99:**1574–1579.
19. **Homonylo-McGavin, M. K., and S. F. Lee.** 1996. Role of the C terminus in antigen P1 surface localization in *Streptococcus mutans* and two related cocci. J. Bacteriol. **178:**801–807.
20. **Jacob, A. E., and S. J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. **117:**360–372.
21. **Jonquieres, R., H. Bierne, F. Fiedler, P. Gounon, and P. Cossart.** 1999. Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of gram-positive bacteria. Mol. Microbiol. **34:**902–914.
22. **Jonquieres, R., J. Pizarro-Cerda, and P. Cossart.** 2001. Synergy between the N- and C-terminal domains of InlB for efficient invasion of non-phagocytic cells by *Listeria monocytogenes*. Mol. Microbiol. **42:**955–965.
23. **Joris, B., S. Englebert, C.-P. Chu, R. Kariyama, L. Daneo-Moore, G. D. Shockman, and J.-M. Ghuysen.** 1992. Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin. FEMS Microbiol. Lett. **91:**257–264.
24. **Kayaoglu, G., and D. Orstavik.** 2004. Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. Crit. Rev. Oral Biol. Med. **15:**308–320.
25. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. USA **100:**1990–1995.
26. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709–712.
27. **Le Loir, Y., A. Gruss, S. D. Ehrlich, and P. Langella.** 1998. A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. J. Bacteriol. **180:**1895–1903.
28. **Le Loir, Y., S. Nouaille, J. Commissaire, L. Bretigny, A. Gruss, and P. Langella.** 2001. Signal peptide and propeptide optimization for heterologous protein secretion in *Lactococcus lactis*. Appl. Environ. Microbiol. **67:**4119–4127.
29. **Lepage, E., S. Brinster, C. Caron, C. Ducroix-Crepy, L. Rigottier-Gois, G. Dunny, C. Hennequet-Antier, and P. Serror.** 2006. Comparative genomic hybridization analysis of *Enterococcus faecalis*: identification of genes absent from food strains. J. Bacteriol. **188:**6858–6868.
30. **Liebl, W., A. J. Sinskey, and K. H. Schleifer.** 1992. Expression, secretion, and processing of staphylococcal nuclease by *Corynebacterium glutamicum*. J. Bacteriol. **174:**1854–1861.
31. **Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss.** 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. J. Bacteriol. **178:**931–935.
32. **Mesnage, S., E. Tosi-Couture, M. Mock, and A. Fouet.** 1999. The S-layer homology domain as a means for anchoring heterologous proteins on the cell surface of *Bacillus anthracis*. J. Appl. Microbiol. **87:**256–260.
33. **Milohanic, E., R. Jonquieres, P. Cossart, P. Berche, and J. L. Gaillard.** 2001. The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. Mol. Microbiol. **39:**1212–1224.
34. **Navarre, W. W., and O. Schneewind.** 1994. Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. Mol. Microbiol. **14:**115–121.
35. **Navarre, W. W., and O. Schneewind.** 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol. Mol. Biol. Rev. **63:**174–229.
36. **Pages, J. M., J. Anba, A. Bernadac, H. Shinagawa, A. Nakata, and C. Lazdunski.** 1984. Normal precursors of periplasmic proteins accumulated in the cytoplasm are not exported post-translationally in *Escherichia coli*. Eur. J. Biochem. **143:**499–505.
37. **Paterson, G. K., and T. J. Mitchell.** 2006. The role of *Streptococcus pneumoniae* sortase A in colonisation and pathogenesis. Microbes Infect. **8:**145–153.
38. **Paulsen, I. T., L. Banerjei, G. S. Myers, K. E. Nelson, R. Seshadri, T. D.**

Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty, and C. M. Fraser. 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299:** 2071–2074.

39. **Piard, J. C., I. Hautefort, V. A. Fischetti, S. D. Ehrlich, M. Fons, and A. Gruss.** 1997. Cell wall anchoring of the *Streptococcus pyogenes* M6 protein in various lactic acid bacteria. J. Bacteriol. **179:**3068–3072.
40. **Poquet, I., S. D. Ehrlich, and A. Gruss.** 1998. An export-specific reporter designed for gram-positive bacteria: application to *Lactococcus lactis*. J. Bacteriol. **180:**1904–1912.
41. **Ries, W., C. Hotzy, I. Schocher, U. B. Sleytr, and M. Sara.** 1997. Evidence that the N-terminal part of the S-layer protein from *Bacillus stearothermophilus* PV72/p2 recognizes a secondary cell wall polymer. J. Bacteriol. **179:**3892–3898.
42. **Rince, A., J. C. Giard, V. Pichereau, S. Flahaut, and Y. Auffray.** 2001. Identification and characterization of *gsp65*, an organic hydroperoxide resistance (*ohr*) gene encoding a general stress protein in *Enterococcus faecalis*. J. Bacteriol. **183:**1482–1488.
43. **Roccourt, J., F. Grimont, P. A. D. Grimont, and H. P. R. Seeliger.** 1982. DNA relatedness among serovars of *Listeria monocytogenes sensu lato*. Curr. Microbiol. **7:**383–388.
44. **Ruhland, G. J., M. Hellwig, G. Wanner, and F. Fiedler.** 1993. Cell-surface location of *Listeria*-specific protein p60—detection of *Listeria* cells by indirect immunofluorescence. J. Gen. Microbiol. **139:**609–616.
45. **Sabet, C., M. Lecuit, D. Cabanes, P. Cossart, and H. Bierne.** 2005. LPXTG protein InlJ, a newly identified internalin involved in *Listeria monocytogenes* virulence. Infect. Immun. **73:**6912–6922.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
47. **Schachtsiek, M., W. P. Hammes, and C. Hertel.** 2004. Characterization of *Lactobacillus coryniformis* DSM 20001^T surface protein Cpf mediating coaggregation with and aggregation among pathogens. Appl. Environ. Microbiol. **70:**7078–7085.
48. **Schneewind, O., A. Fowler, and K. F. Faull.** 1995. Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. Science **268:**103–106.
49. **Siezen, R., J. Boekhorst, L. Muscariello, D. Molenaar, B. Renckens, and M. Kleerebezem.** 2006. Lactobacillus plantarum gene clusters encoding putative cell-surface protein complexes for carbohydrate utilization are conserved in specific gram-positive bacteria. BMC Genomics **7:**126.
50. **Simon, D., A. Rouault, and M. C. Chopin.** 1986. High-efficiency transformation of *Streptococcus lactis* protoplasts by plasmid DNA. Appl. Environ. Microbiol. **52:**394–395.
51. **Steen, A., G. Buist, K. J. Leenhouts, M. El Khattabi, F. Grijpstra, A. L. Zomer, G. Venema, O. P. Kuipers, and J. Kok.** 2003. Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. J. Biol. Chem. **278:**23874–23881.
52. **Sutcliffe, I. C., and R. R. Russell.** 1995. Lipoproteins of gram-positive bacteria. J. Bacteriol. **177:**1123–1128.
53. **Tendolkar, P. M., A. S. Baghdayan, and N. Shankar.** 2003. Pathogenic enterococci: new developments in the 21st century. Cell. Mol. Life Sci. **60:**2622–2636.
54. **Teng, F., K. D. Jacques-Palaz, G. M. Weinstock, and B. E. Murray.** 2002. Evidence that the enterococcal polysaccharide antigen gene (*epa*) cluster is widespread in *Enterococcus faecalis* and influences resistance to phagocytic killing of *E. faecalis*. Infect. Immun. **70:**2010–2015.
55. **Tjalsma, H., and J. M. van Dijl.** 2005. Proteomics-based consensus prediction of protein retention in a bacterial membrane. Proteomics **5:**4472–4482.
56. **Waldemarsson, J., T. Areschoug, G. Lindahl, and E. Johnsson.** 2006. The streptococcal Blr and Slr proteins define a family of surface proteins with leucine-rich repeats: camouflaging by other surface structures. J. Bacteriol. **188:**378–388.
57. **Yamamoto, H., S. Kurosawa, and J. Sekiguchi.** 2003. Localization of the vegetative cell wall hydrolases LytC, LytE, and LytF on the *Bacillus subtilis* cell surface and stability of these enzymes to cell wall-bound or extracellular proteases. J. Bacteriol. **185:**6666–6677.
58. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.