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# TgaA, a VirB1-Like Component Belonging to a Putative Type IV Secretion System of *Bifidobacterium bifidum* MIMBb75

Simone Guglielmetti,<sup>a</sup> Silvia Balzaretto,<sup>a</sup> Valentina Taverniti,<sup>a</sup> Matteo Miriani,<sup>a</sup> Christian Milani,<sup>b</sup> Alessio Scarafoni,<sup>a</sup> Silvia Corona,<sup>c</sup> Alessandro Ciranna,<sup>d</sup> Stefania Arioli,<sup>a</sup> Ville Santala,<sup>d</sup> Stefania Iametti,<sup>a</sup> Francesco Bonomi,<sup>a</sup> Marco Ventura,<sup>b</sup> Diego Mora,<sup>a</sup> Matti Karp<sup>d</sup>

Department of Food Environmental and Nutritional Sciences, Università degli Studi di Milano, Milan, Italy<sup>a</sup>; Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parma, Italy<sup>b</sup>; IRCCS Policlinico San Matteo, Pavia, Italy<sup>c</sup>; Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland<sup>d</sup>

*Bifidobacterium bifidum* MIMBb75 is a human intestinal isolate demonstrated to be interactive with the host and efficacious as a probiotic. However, the molecular biology of this microorganism is yet largely unknown. For this reason, we undertook whole-genome sequencing of *B. bifidum* MIMBb75 to identify potential genetic factors that would explain the metabolic and probiotic attributes of this bacterium. Comparative genomic analysis revealed a 45-kb chromosomal region that comprises 19 putative genes coding for a potential type IV secretion system (T4SS). Thus, we undertook the initial characterization of this genetic region by studying the putative *virB1*-like gene, named *tgaA*. Gene *tgaA* encodes a peptidoglycan lytic enzyme containing two active domains: lytic murein transglycosylase (LT, cd00254.3) and cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP, pfam05257.4). By means of several *in vitro* assays, we experimentally confirmed that protein TgaA, consistent with its computationally assigned role, has peptidoglycan lytic activity, which is principally associated to the LT domain. Furthermore, immunofluorescence and immunogold labeling showed that the protein TgaA is abundantly expressed on the cell surface of *B. bifidum* MIMBb75. According to the literature, the T4SSs, which have not been characterized before in bifidobacteria, can have important implications for bacterial cell-to-cell communication as well as cross talk with host cells, justifying the interest for further studies aimed at the investigation of this genetic region.

**B**ifidobacteria are Gram-positive members of the human gut microbiota that include species typically representing the dominant colonic bacteria in breast-fed infants (1, 2). Together with lactobacilli, bifidobacteria are the microorganisms most widely exploited in probiotic formulations because of their various health-promoting properties. In this regard, the ability of *Bifidobacterium* to interact with the host has been demonstrated to be species and strain specific (3–5). Notably, members of the species *Bifidobacterium bifidum* are capable of interaction with the host, including degradation of mucin (6, 7), metabolism of human milk oligosaccharides (8), and modulation of host immune responses (9–11). Therefore, the species *B. bifidum* represents a good source of bacterial strains to be employed for the development of novel effective probiotic (12) or paraprobiotic (13) products.

*B. bifidum* MIMBb75 is a human intestinal isolate with a marked ability to adhere to intestinal mucosae (6, 8), transiently colonize murine intestine, and impact the resident microbial communities (14). Furthermore, the probiotic potential of this strain was evidenced in a clinical trial in which daily consumption of *B. bifidum* MIMBb75 significantly alleviated the global symptoms of irritable bowel syndrome and improved the quality of life (15). Though MIMBb75 is efficacious as a probiotic, the molecular mechanisms underlying such interaction are yet largely unknown. For this reason, we undertook whole-genome sequencing of MIMBb75 to identify potential genetic factors useful to elucidate the metabolic and probiotic potentialities of this bacterium.

Type IV secretion systems (T4SSs) are large bacterial protein complexes that transport macromolecules across the cell envelope (16). Reportedly, T4SSs are involved in naked DNA uptake and release (17), conjugation (18), and delivery of effector molecules (DNA and/or protein) into eukaryotic host cells (19). To support

these activities, the proteins constituting T4SSs include (i) peptidoglycan lytic enzymes, (ii) energizing (motor) components, (iii) scaffold/putative core components, and (iv) surface factors/adhesins (20). T4SSs have been studied in detail in certain Gram-negative bacteria, which use these conjugation apparatuses for the translocation of genetic material and effector proteins into other bacteria or eukaryotic host cells (19). In contrast, T4SSs described so far in Gram-positive bacteria appear to be exclusively dedicated to conjugative DNA transfer, as demonstrated by the presence of members of an additional family of DNA processing enzymes not present in T4SSs participating in protein effector transport (20). Furthermore, Gram-positive conjugative T4SSs do not produce pilus filaments but contain surface proteins that operate as adhesins (21). It is plausible that T4SSs in Gram-positive cells serve additional and yet unknown purposes.

Although identified in the genome of numerous Gram-positive bacteria, T4SSs have not been characterized in bifidobacteria. In this regard, here we show the identification by comparative genomics of a genetic region putatively coding for a type IV secretion system in *B. bifidum* MIMBb75. As a first step in the characterization of the putative T4SS of strain MIMBb75, in this study,

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Address correspondence to Simone Guglielmetti, simone.guglielmetti@unimi.it.

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we describe the molecular analysis of one of its constitutive elements, namely, the putative VirB1-like peptidoglycan-lytic component encoded by gene *tgaA*. In addition, gene *tgaA* attracted our attention because its product probably is located on the outer surface of the bacterial cells and therefore has major chances to interact with host cells.

## MATERIALS AND METHODS

**Bifidobacterial culture conditions.** *Bifidobacterium* strains were grown under anaerobic condition (Anaerocult A System; Merck, Darmstadt, Germany) at 37°C in prereduced DeMan-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Inc., Detroit, MI) supplemented with 0.05% L-cysteine hydrochloride (cMRS; Sigma-Aldrich, St. Louis, MO).

**Genome sequencing, sequence annotation, and comparative analysis.** The genome sequence of *B. bifidum* MIMBb75 was determined by the use of an Illumina HiSeq 2000 system with paired-end and shotgun libraries. A total of 5,722,028 reads with an average length of 35 bp were assembled into 133 contigs using SOAPde novo, version 1.04, software (22). Protein-encoding open reading frames (ORFs) were predicted using a combination of Prodigal (23) and BLASTX (24) for comparative analysis. Results of the gene-finder program were combined manually with data from BLASTP (25) analysis against a nonredundant protein database provided by the National Center for Biotechnology Information. The combined results were inspected by Artemis (26), which was used for a manual editing effort to verify and, if necessary, to redefine the start of each predicted coding region or to remove or add coding regions within the 45-kb region containing the *tgaA* gene (i.e., the putative type IV secretion system region [T4SS]). The TMHMM server, version 2.0 (27), was used to predict transmembrane sequences, and SignalP (28) was used for the prediction of signal peptides. Whole-genome sequence alignments for similarity were performed at the DNA level using LAST (<http://last.cbrc.jp/>).

***tgaA* gene expression analysis.** Total RNA was extracted from *B. bifidum* MIMBb75 collected at the early logarithmic (optical density at 600 nm [OD<sub>600</sub>] of 0.5 to 1), late logarithmic (OD<sub>600</sub> of 2 to 2.5), and stationary (OD<sub>600</sub> of 2.5 to 3) growth phases as previously described (29). In brief, a volume of cMRS broth culture containing 10<sup>9</sup> cells was collected and centrifuged (at 4°C for 1 min at 8,000 × g). The pellet was resuspended in 1 ml of PureZol (Bio-Rad Laboratories, Milan, Italy) and subjected to a mechanical disruption using a Precellys bead beater (Advanced Biotech Italia s.r.l., Seveso, Italy). Total RNA extraction was performed with an Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad Laboratories), and samples were additionally treated with DNase I (Sigma-Aldrich) for 30 min at room temperature. Subsequently, iScript™ cDNA Synthesis (Bio-Rad Laboratories) was used to obtain cDNA from 1 μg of RNA. Finally, PCR was carried out using a volume of cDNA solution corresponding to 15 ng of initial RNA in a total reaction volume of 25 μl using DreamTaq DNA polymerase (Fermentas, Vilnius, Lithuania). The expression level of the gene *tgaA* was assessed with primers Cha-F (5'-CGCGACGTGATCTGCTTCGA-3') and Cha-R (5'-CCGATTGGCTGATGAGATC-3'), which allow the amplification of a 115-bp *tgaA* internal fragment. Internal normalization was obtained through the amplification of an approximately 500-bp fragment from the 16S rRNA gene with primers g-Bifid-F (5'-CTCCTGGAAACGGGTGG-3') and g-Bifid-R (5'-GGTGTCTTCCCGATATCTACA-3'). Annealing temperatures in the PCR were 60°C and 55°C for the Cha and g-Bifid primers, respectively.

**Expression of TgaA-derived recombinant proteins in *Escherichia coli*.** All enzymes and reagents for molecular biology reactions (polymerases, T4 ligase, and restriction enzymes) were from Fermentas or New England BioLabs (EuroClone S.p.A., Pero, Italy). Purification of PCR fragments was performed with UltraClean PCR Cleanup (MoBio Laboratories/Cabru s.a.s., Arcore, Italy). Plasmid extraction from *E. coli* was carried out with a QIAprep Spin miniprep kit (Qiagen s.r.l., Milan, Italy). DNA fragments in agarose gel were extracted and purified with a

QIAquick gel extraction kit (Qiagen). Antibiotics for the selection of plasmids were from Sigma-Aldrich (St. Louis, MO). For the preparation of vector pAK-tga, the gene *tgaA* (without the original signal peptide [SP]) was amplified by PCR from *B. bifidum* MIMBb75 chromosomal DNA with primers tgaFOR-MA (5'-TTACTCGCGGCCAGCCGGCCATGGCGACGGAATCCACGGTC-3') and tgaREV (5'-ATGGTGATCGGCCCGGAGGCCGTGTGAATGAAGCTGACGCT-3'). The amplicon was digested with SfiI restriction enzyme (SfiI restriction sites in the primers are underlined) and ligated in the same restriction site of vector pAK400 (30), yielding a transcriptional fusion of the *tgaA* amplicon with an *E. coli pelB* signal sequence (SP<sub>pelB</sub>) that was present in vector pAK400. The ligation mix was used to transform *E. coli* JM109 by electroporation according to standard protocols. Recombinants of *E. coli* JM109 were cultured at 37°C in Super Optimal Broth (SOB) medium supplemented with 25 μg ml<sup>-1</sup> chloramphenicol. Once expressed, the signal peptide SP<sub>pelB</sub> allows the export of the recombinant protein to the periplasm of *E. coli* cells. For the overexpression of TgaA recombinant proteins, we prepared three plasmids based on expression vector pET26b(+) (Novagen/Merck KGaA, Darmstadt, Germany) and the expression host *E. coli* BL21(DE3) pLysS (Promega, Madison, WI, USA). In detail, gene *tgaA* (without the original signal peptide) was amplified by PCR from *B. bifidum* MIMBb75 chromosomal DNA with primers TgaNdeF (5'-CCTTGGAAACATATGAGCGAATCCACGGTCG-3') and TgaHindR (5'-TCTAGGAAAAGCTTGTGATGAAGCTGACG-3'), which include restriction sites NdeI and BamHI, respectively (underlined). The resulting amplicon was digested and ligated in the same restriction sites of vector pET26b(+), yielding a transcriptional fusion with a six-His tag at the 3' terminus of the gene. The resulting vector, named pET-Tga, was subsequently digested with Bsp19I, yielding two DNA fragments. The larger fragment (containing the vector replicon) was separated in and purified from agarose gel and finally self-ligated, yielding vector pET-ΔCHAP (where CHAP is cysteine- and histidine-dependent amidohydrolase/peptidase, pfam05257.4). Afterwards, we prepared vector pET-ΔLT (where LT is lytic murein transglycosylase, cd00254.3) by inverse PCR amplification of plasmid pET-Tga with primers dLT-F (5'-ATACCGGAATTCACGGAGACCACCA-3') and dLT-R (5'-TTCCAATTGAATTCCTGGTCGATCT-3'), followed by digestion of the amplicon with EcoRI (the restriction site is underlined in primer sequences), and self-ligation. The recombinants of *E. coli* BL21(DE3) pLysS were cultured at 37°C in the presence of 25 μg ml<sup>-1</sup> chloramphenicol (selection for pLysS plasmid) and 25 μg ml<sup>-1</sup> kanamycin (selection for pET vectors). The absence of mutations or recombinations in the obtained plasmids was confirmed by sequencing analysis. Vectors pAK-Tga, pET-ΔCHAP, and pET-ΔLT were used for the overexpression of the TgaA-derived recombinant proteins.

**Analysis of intracellular accumulation of cell wall breakdown products.** In order to determine the accumulation of fragments deriving from cell wall lysis in *E. coli* cells, we used the bioluminescence reporter system based on vector pBlaLux1 and *E. coli* SNO301 (31). Briefly, we transformed by electroporation the cell reporter system with vector pAK-Tga. The expression of TgaA recombinant protein from pAK-Tga was obtained by adding isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich) to the cell culture. To assess cell wall lysis, immediately after IPTG addition, the broth cultures were aliquoted in a microtiter plate and incubated at 37°C in a luminometer (Chameleon Multilabel Detection Platform; Hidex OY, Turku, Finland) that was set to automatically measure light emission every 5 min for several hours.

**Protein overproduction.** Vectors pAK-Tga, pET-ΔCHAP, and pET-ΔLT were used to overexpress in *E. coli* the recombinant proteins ΔSP-TgaA-His (containing both domains of TgaA protein; also named SP<sub>pelB</sub>-TgaA here and rTgaA for recombinant TgaA in an accompanying paper [32]), protein ΔSP-TgaA-ΔCHAP-His (containing only the LT domain; rLT), and ΔSP-TgaA-ΔLT-His (containing only the CHAP domain; rCHAP), respectively. To this aim, recombinant *E. coli* strains were grown over night in 2× YT (yeast extract, tryptone) broth at 37°C and then

inoculated in 200 ml of the same medium (1:100 inoculum). After 2 h of incubation under strong agitation at 37°C, 1 mM IPTG was added, and incubation was prolonged for 4 h. Afterwards, cells were recovered by centrifugation, weighed, and treated for protein extraction under denaturing conditions with PerfectPro Ni-nitrilotriacetic acid (NTA) agarose (5 Prime; Eppendorf Italia s.r.l., Milan, Italy) according to the manufacturer's instructions.

**Detection of peptidoglycan lytic activity by renaturing SDS-PAGE.** Renaturing SDS-PAGE was performed as described by Mora et al. (33). In brief, autoclaved cells of *Micrococcus luteus* ATCC 4698 (Sigma-Aldrich) were polymerized using a 12% SDS-PAGE gel at a concentration of 0.2% (wt/vol). Sample preparation was carried out by mixing 5 µg of recombinant protein with 30 µl of SDS-PAGE denaturing loading buffer. After electrophoresis, gels were soaked in 200 ml of sterilized, distilled water for 30 min and then in 200 ml of 25 mmol liter<sup>-1</sup> Tris-HCl (pH 7) containing 1% (wt/vol) Triton X-100 for 12 to 36 h. Bands of lytic activity were visualized by staining the gel with 1% (wt/vol) methylene blue (Sigma) in 0.1% (wt/vol) KOH and subsequent destaining in sterilized distilled water.

**Generation of ΔSP-TgaA-ΔLT-His-specific antibodies.** An antibody against the recombinant protein rCHAP was raised in rabbits by Primm s.r.l. (Milan, Italy). Briefly, after preimmune serum collection, an initial immunization was followed by a second immunization at day 21, a third immunization at day 28, a bleed step and enzyme-linked immunosorbent assay (ELISA) at day 35, and a fourth immunization at day 37. Then, a final serum sampling and affinity purification of antibodies were carried out.

**Preparation and analysis of immunofluorescent *B. bifidum* MIMBb75.** *B. bifidum* MIMBb75 cells were cultivated until early stationary growth phase, harvested by centrifugation, washed once with deionized sterile water, and incubated at room temperature for 10 min with ΔSP-TgaA-ΔLT-His antiserum (1:100 diluted in deionized water). Subsequently, bacterial cells were centrifuged, resuspended in a solution of Cy5-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) (1:10 diluted in deionized water), and incubated for 10 min at room temperature in the dark. Afterwards, stained cells were visualized with a fluorescence optical digital microscope (Leica DM1000; Leica Microsystems, Wetzlar, Germany) at a magnification of ×1,000.

**Electron microscopy and immunogold labeling.** Bifidobacterial cells were cultivated in broth to stationary phase (15 to 18 h of cultivation) or on agar plates of cMRS medium. Bacterial cells were collected, and the resulting pellet was processed for transmission electron microscopy. Sample preparation was performed according to Arioli et al. (34). In brief, cells were fixed in 2.5% glutaraldehyde and later postfixed with 1% osmium tetroxide (in 0.1 M cacodylate buffer, pH 7.2) for 2 h at room temperature. After the remaining osmium tetroxide was eliminated, the samples were dehydrated in a graduated cold ethanol series (35 to 100%); each step was performed for about 10 to 15 min at room temperature. The fixed cells were embedded in Epon 812. Blocks were cut with an ultramicrotome and collected on nickel grids. Sections were poststained with 5% uranyl acetate for 5 min at room temperature and treated with lead citrate for 1 min. Sections were observed and photographed with a Philips CM 12 electron microscope and a Zeiss 900 (Carl Zeiss S.p.A., Arese, Milan, Italy). Bacterial cells were also observed after direct staining (intact-cell analysis). For immunogold electron microscopy analysis, samples were treated with anti-ΔSP-TgaA-ΔLT-His rabbit immune serum (or preimmune serum), and the antibodies were then labeled with protein A-conjugated gold particles (10 nm). Finally, the samples were negatively stained with uranyl acetate and lead citrate as indicated above and examined under an electron microscope.

**Nucleotide sequence accession number.** The DNA sequence of the T4SS region from *B. bifidum* MIMBb75 has been deposited in the EMBL database under the accession number [HG794715](https://www.ebi.ac.uk/EMBL/nuccore/HG794715).

## RESULTS AND DISCUSSION

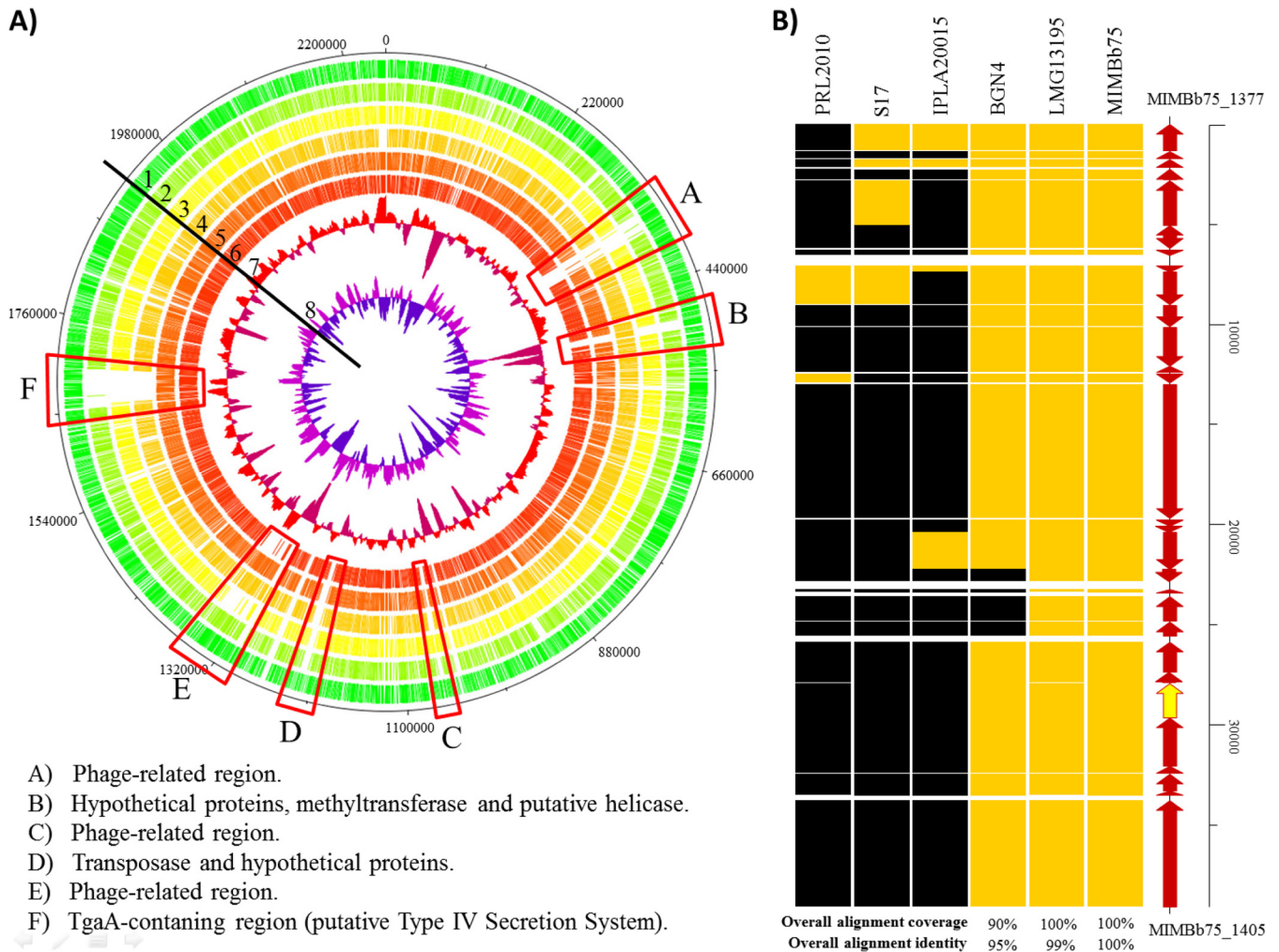
**In silico analysis of the *B. bifidum* MIMBb75 genome reveals a chromosomal region putatively coding for a type IV secretion system.** We generated a draft genome sequence of *Bifidobacterium bifidum* MIMBb75 consisting of 133 contigs, which in total contained 2.25 Mbp. The guanine and cytosine (G+C) content was calculated to be 62.76%, which is consistent with that of other *B. bifidum* genomes (7).

Comparative genomics revealed that more than 90% of the putative coding sequences identified in MIMBb75's contigs share high sequence homology with all of the *B. bifidum* genomes available so far (GenBank accession numbers: strain BGN4, [NC\\_017999](https://www.ncbi.nlm.nih.gov/nuccore/NC_017999); PRL2010, [NC\\_014638](https://www.ncbi.nlm.nih.gov/nuccore/NC_014638); S17, [NC\\_014616](https://www.ncbi.nlm.nih.gov/nuccore/NC_014616); IPLA20015, from [AMPM01000001](https://www.ncbi.nlm.nih.gov/nuccore/AMPM01000001) to [AMPM01000050](https://www.ncbi.nlm.nih.gov/nuccore/AMPM01000050); LMG13195, from [AMPL01000001](https://www.ncbi.nlm.nih.gov/nuccore/AMPL01000001) to [AMPL01000050](https://www.ncbi.nlm.nih.gov/nuccore/AMPL01000050)). The only exceptions were six chromosomal regions, three of which are related to putative integrated phages or phage remnants (Fig. 1A, regions A, C, and E) and two of which are related to DNA modification enzymes (regions B and D); the other non-conserved genetic locus (region F) consists of a 45-kb region, which includes the putative genes of a type IV secretion system (T4SS).

Even though T4SSs are widespread among all bacterial phyla, their molecular component functions have been studied in depth only within Gram-negative bacteria, such as the *vir* region of *Agrobacterium tumefaciens* (35) and the *cag* type IV secretion apparatus of *Helicobacter pylori* (36). The gene nomenclature of the 43-kbp *Agrobacterium vir* T4SS region has been used to refer also to T4SSs identified in Gram-positive bacteria (37, 38).

We found 19 putative genes (Fig. 2A, ORFs 1 to 19) in the predicted T4SS genetic locus of *B. bifidum* MIMBb75. These putative genes include the minimal components of the secretory system in Gram-positive bacteria (VirB1-, VirB4-, VirB6-, and VirD4-like proteins) and the proteins needed to support the conjugative transfer of DNA (37, 38). Specifically, we found in MIMBb75 seven genes whose products contain conserved domains associated to *vir* molecular components of the *Agrobacterium tumefaciens* T4SS (Fig. 2A): ORF4 putatively codes for a VirD2-like relaxase/mobilization protein (conserved domain pfam03432) (Fig. 3, D2), whereas the polypeptide deduced from ORF8 shows the conserved domains cd01126 and pfam12696, which characterize TraG/TraD/VirD4-like proteins (Fig. 3, D4); ORF11 shares homology with a VirB1-type lytic transglycosylase, functioning to degrade peptidoglycan to facilitate insertion of the Gram-positive bacterial type IV secretion machinery into the cell envelope (38–40); the ORF12-encoded peptide contains a sequence homologous to the conserved domains pfam12846, TIGR02746, and TIGR00929, which are typical of TrbE/VirB4-like proteins (Fig. 3, B4). The VirB4- and VirD4-like enzymes of the T4SSs are coupling protein NTPases that play essential roles in supplying the energy for substrate translocation and apparatus assembly (31, 36, 41). ORF13 putatively codes for VirB6-like proteins (Fig. 3, B6), which constitute a component of the transmembrane channel (16), whereas ORF15 is the potential gene for VirC1 protein, a factor required for efficient DNA transfer (42). Therefore, the 45-kb genetic locus of *B. bifidum* MIMBb75 seems to have the main elements necessary for a functional type IV secretion system (Fig. 3 describes the potential mechanism of DNA transfer by the putative T4SS of *B. bifidum* MIMBb75).



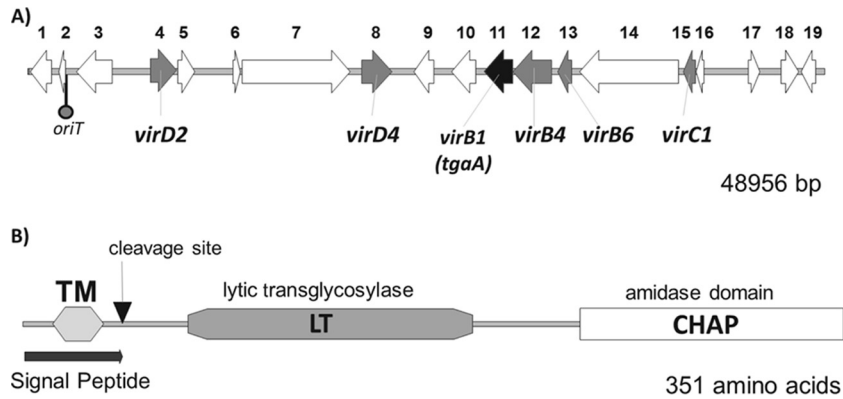


**FIG 1** Comparative genomic analysis of *B. bifidum* MIMBb75 with other complete genome sequences of *B. bifidum* strains. (A) Circular genome atlas of *B. bifidum* MIMBb75 (circle 1) with mapped orthologues (defined as reciprocal best BLASTP hits with more than 30% identity over at least 80% of both protein lengths) in six publicly available *B. bifidum* genomes: *B. bifidum* PRL2010 (circle 2), *B. bifidum* S17 (circle 3), *B. bifidum* IPLA20015 (circle 4), *B. bifidum* LMG13195 (circle 5), and *B. bifidum* BGN4 (circle 6). Circle 6 shows the GC plot, and circle 8 shows the GC skew. Highlighted in the atlas are the main regions of difference between the genomes. (B) Heatmap showing genes encompassing the *tgaA*-containing region and conserved among the other sequenced *B. bifidum* genomes. Yellow indicates the chromosomal regions homologous to the putative MIMBb75 T4SS, whereas black indicates the absence of homology.

**ORF11 encodes TgaA, a putative VirB1-like enzyme.** With the aim of characterizing the putative T4SS of *B. bifidum* MIMBb75, in this study we initially investigated ORF11, which potentially corresponds to a VirB1-like protein. In the T4SS, VirB1 is a protein that harbors a lysozyme-like structural fold. Consistently, we found that the protein encoded by ORF11 contains two conserved domains involved in the metabolism of the bacterial cell wall, lytic murein transglycosylase (LT, cd00254.3) and cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP, pfam05257.4) (Fig. 2B), according to an organization that resembles the structure of autolytic enzymes (43, 44). Bacterial lytic transglycosylases degrade murein by nonhydrolytic cleavage of the  $\beta$ -1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine and are part of the autolytic enzymes involved in controlled turnover of the bacterial cell wall (45, 46). The CHAP domain is found in a wide range of protein architectures, often in association with other domains that cleave peptidoglycan (41, 47), and presumably shows *N*-acetylmuramoyl-L-

alanine amidase activity (48). Consequently, the putative gene corresponding to ORF11 was named *tgaA* for transglycosylase amidase gene A.

**Distribution of TgaA protein in bifidobacteria.** The overall structure of the TgaA protein (i.e., the LT conserved domain cd00254.3, followed by the CHAP domain pfam05257.4 at the C terminus) was found in eight *Bifidobacterium* coding sequences available in GenBank, including three putative proteins belonging to the species *B. breve* and two belonging to *B. bifidum*. Besides the above-mentioned eight sequences, the LT cassette was found in only one additional putative protein (YP\_004209100 from *B. longum* subsp. *infantis* 157F) (see Fig. S4 in the supplemental material), whereas a conserved domain of the CHAP superfamily was identified in numerous bifidobacterial putative proteins and particularly in proteins where no other conserved domains are identified (see Fig. S4). The CHAP domain is, therefore, widespread among bifidobacteria (18 species), indicating that its enzymatic activity could be involved in cellular functions of fundamental

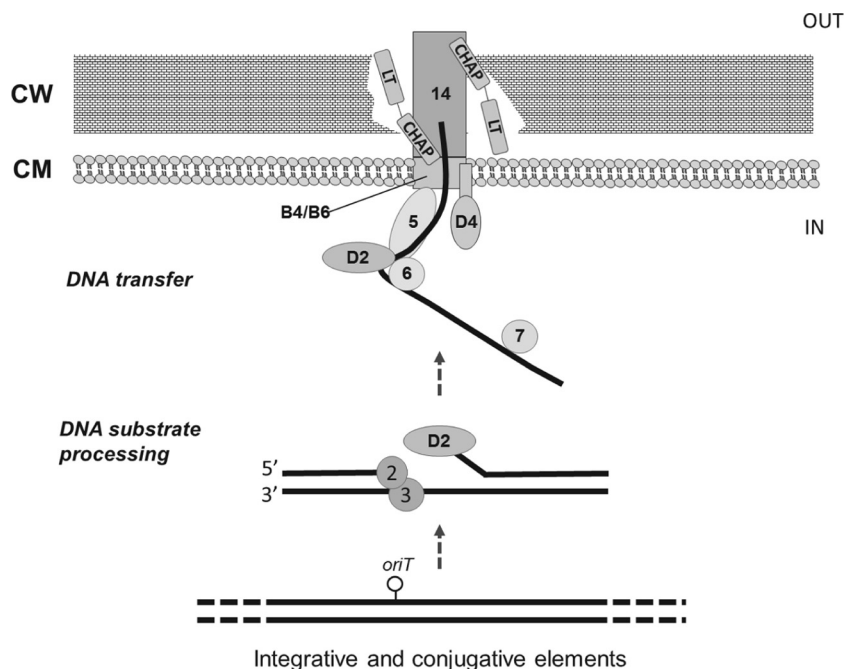


**FIG 2** Organization of the putative type IV secretion system genetic region of *Bifidobacterium bifidum* MIMBb75 (A) and structural organization of the TgaA protein (B). ORFs indicated in the map in panel A putatively code for the following proteins (according to a BLASTP search): 1, HipA-like domain-containing protein (PF07805); 2, helix-turn-helix XRE family-like protein (HipB); 3, DNA topoisomerase III; 4, relaxase (VirD2); 5, mobilization protein (MobC); 6, conserved transposon-encoded protein (TnpV); 7, methyl transferase type 11; 8, VirD4 ATPase (TraG/TraD family protein); 9, HipB transcriptional regulator (HTH XRE); 10, molecular chaperone (containing the pfam02459 motif); 11, TgaA (VirB1); 12, transfer complex protein (VirB4); 13, plasmid conjugal transfer protein (VirB6/TrbL); 14, aggregation substance (containing domains pfam10698 and TIGR04228); 15, ParA ATPase (VirC1); 16, transcription regulator WhiB-related protein; 17, acetyltransferase (GNAT) family; 18, HTH XRE (HipB, containing the domain cd00093); 19, HAD-type hydrolase (COG0561). ORFs putatively coding for hypothetical proteins have not been included. *oriT*, putative origin of transfer. TM, transmembrane region.

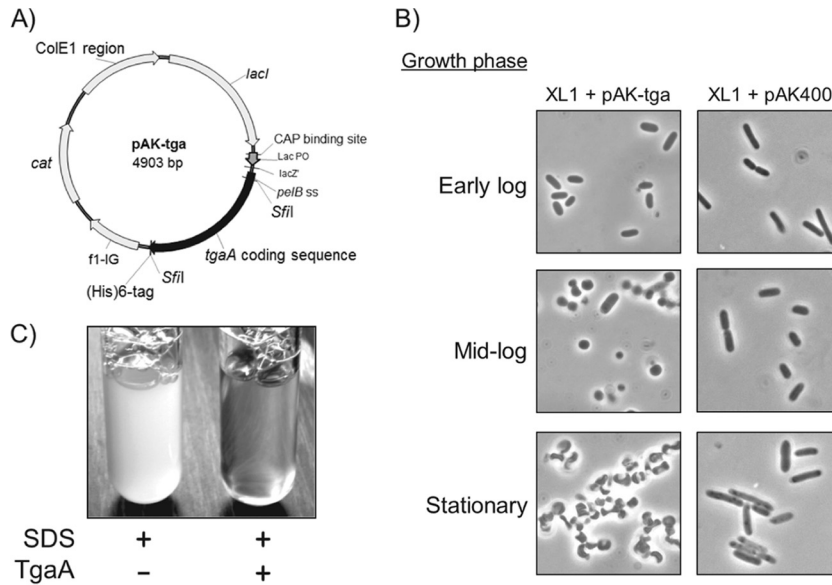
importance for cell wall metabolism (44). Interestingly, about 14 protein spots in the extracellular proteome of *B. animalis* subsp. *lactis* BB-12 were attributed to BIF\_00825 and BIF\_01398, two proteins containing a CHAP amidase domain (49). In contrast, in our study we failed to detect any significant positive signal when we tested an MIMBb75 cell-free cMRS broth culture using West-

ern blot analysis with anti-CHAP polyclonal antibodies (data not shown).

**TgaA is a peptidoglycan lytic enzyme.** We initially verified by reverse transcription-PCR (RT-PCR) that the *tgaA* gene is expressed by *B. bifidum* MIMBb75 during the initial exponential growth phase (see Fig. S1 in the supplemental material). Subse-

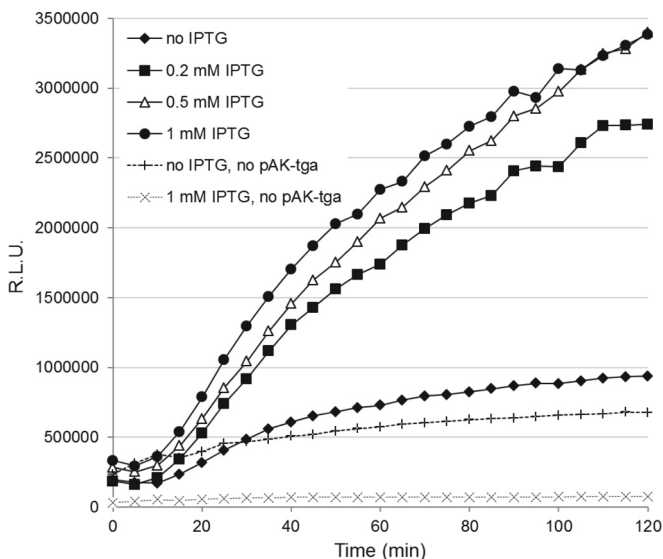


**FIG 3** Proposed mechanism of the putative type IV secretion system (T4SS) of *Bifidobacterium bifidum* MIMBb75. (The illustration is based on the scheme in reference 51.) The numbers and codes of genes and proteins are according to Fig. 2A. CW, cell wall; CM, cell membrane. The T4SS is comprised of conjugation, effector translocator, and DNA release/uptake subfamilies of proteins. For conjugative transfer, DNA substrates are processed through (i) excision from the chromosome by excisionase/integrase enzymes or transposases (e.g., VirD2), (ii) processing of the transfer intermediate at the origin-of-transfer sequence (*oriT*) by the relaxosome (e.g., ORF5 and ORF6), (iii) recruitment of the relaxase T-strand intermediate to the type IV coupling proteins (e.g., VirB4/D4), and (iv) translocation through the T4SS channel (e.g., ORF14).



**FIG 4** Determination of the cell wall lytic activity of TgaA protein. A recombinant form of TgaA ( $SP_{pelB}$ -TgaA) was expressed in *E. coli* XL1 through vector pAK-tga (A), resulting in altered cell morphology after 24 h of growth at 37°C in 2× YT medium (B). After wild-type XL1 cells were incubated at 37°C for 4 h in the presence of 50 μg of purified  $SP_{pelB}$ -TgaA, the addition of 10% SDS to a final concentration of 0.2% determined instantaneous complete cell lysis (C). Vector pAK-tga was obtained by cloning gene *tgaA* in the empty vector pAK400.

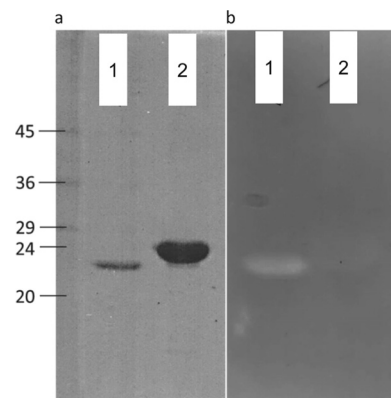
quently, we prepared an engineered *E. coli* construct expressing a recombinant form of TgaA ( $SP_{pelB}$ -TgaA) on IPTG induction in order to biologically confirm the enzymatic activity computationally assigned to the protein TgaA. In  $SP_{pelB}$ -TgaA, the original signal peptide of TgaA was replaced by a *pelB* signal sequence for export at the periplasmic level in *E. coli* (Fig. 4A). As an initial



**FIG 5** Double expression reporter system for the determination of the peptidoglycan lytic activity of TgaA. A dose-dependent increase of bioluminescence by IPTG induction in *E. coli* SNO301 containing pAK-tga and pBlaLux1 was observed. The same strain containing only vector pBlaLux1 was used as a control. The addition of IPTG to *E. coli* SNO301 containing only pBlaLux1 (and not pAK-tga) determined a reduction of the light emission (dotted lines). RLU, relative luminescence units. Each point in the plot represents the mean of the values obtained in one representative experiment conducted in quadruplicates. The variation between the replicates was less than 10%.

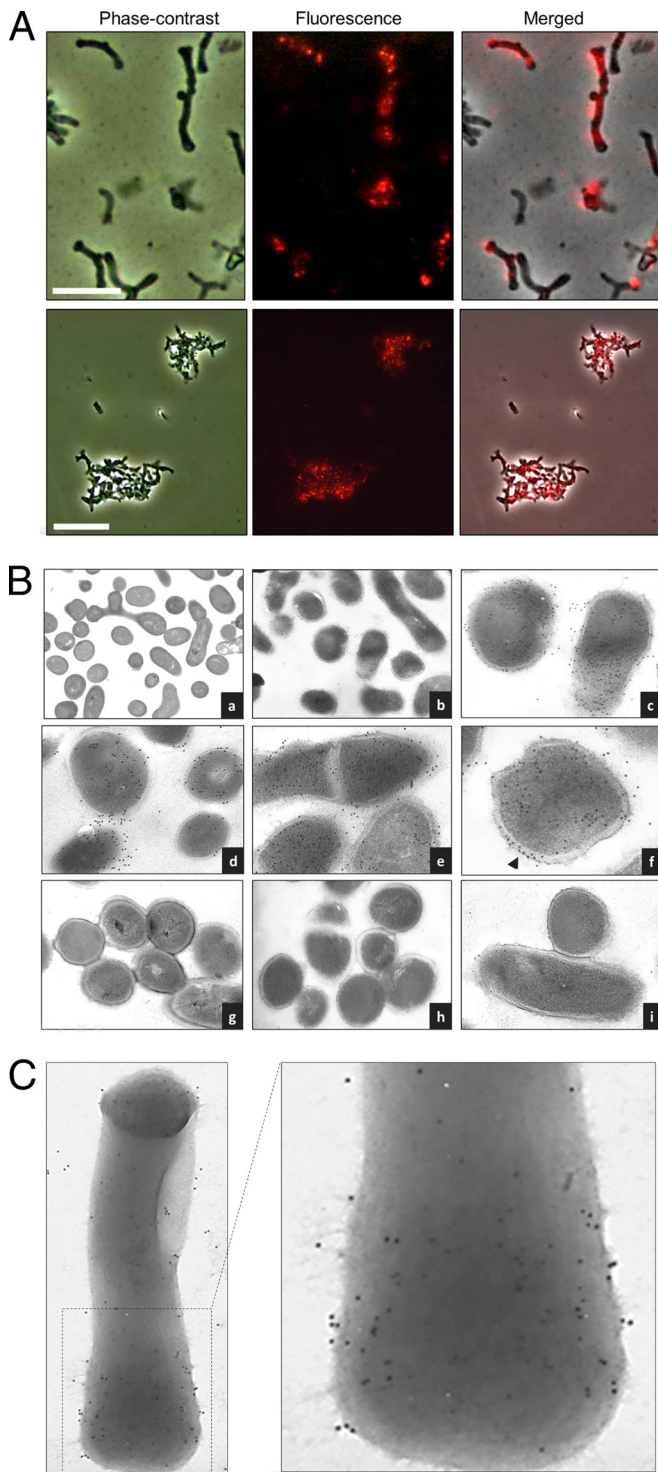
confirmation of TgaA lytic activity, we observed a drastic morphological modification of recombinant *E. coli* cells at the end of the exponential growth phase (Fig. 4B), which was not detected in the same *E. coli* strain harboring the empty vector pAK400. In addition, incubation for a few hours of a suspension of wild-type *E. coli* XL1 with purified  $SP_{pelB}$ -TgaA protein resulted in a complete lysis of the bacterial cells (Fig. 4C).

In a following experiment, we confirmed the lytic activity of TgaA by means of a luminescence reporter system based on vector pBlaLux1 (31). In pBlaLux1, the *luxCDABE* operon of *Photobacterium luminescens* is under the control of the regulatory element *ampR-ampC* of *Citrobacter freundii* ( $P_{ampC}$ ), which was demonstrated to be activated by peptidoglycan fragments (see Fig. S2 in the supplemental material). The introduction of the vector ex-



**FIG 6** SDS-PAGE (a) and renaturing SDS-PAGE containing 0.2% autoclaved *M. luteus* cells (b) of the purified six-His-tagged TgaA-derived recombinant proteins. Lane 1,  $\Delta SP$ -TgaA- $\Delta CHAP$ -His (rLT); lane 2,  $\Delta SP$ -TgaA- $\Delta LT$ -His (rCHAP). The relative molecular masses (in kDa) of standard proteins are indicated on the left.





**FIG 7** Localization of the TgaA protein in *B. bifidum* MIMBb75 cells by immunofluorescence staining (A) and immunogold labeling (B and C). (A) Bacterial cells were probed with  $\Delta$ SP-TgaA- $\Delta$ LT-His antiserum, followed by a reaction with a Cy5-conjugated goat anti-rabbit secondary antibody. Cells were visualized with a fluorescence microscope. Fluorescence, phase-contrast, and merged images of the bacterial cells in the same field are shown. The upper pictures show free bacterial cells (scale bar, 5  $\mu$ m), whereas lower panels show cell aggregates (scale bar, 10  $\mu$ m). (B and C) Bacterial cells were probed with the  $\Delta$ SP-TgaA- $\Delta$ LT-His antiserum, followed by interaction with gold (12-nm)-conjugated goat anti-rabbit IgG, and negatively stained. We tested thin sections of cell-embedded Lowicryl HM20 resin (B) and intact cells (C). Identifications for panel B are as follows: frame a, nonlabeled MIMBb75 cells; frames b to f, *B. bifidum* MIMBb75; frames g to i, *B. bifidum* PRL2010. The arrow in frame f indicates abundant staining of the MIMBb75 cell wall. Bacterial cells were visualized with a transmission electron microscope at a magnification of 23,000 to 60,000.

pressing SP<sub>pelB</sub>-TgaA in the microbial biosensor resulted in a dose-dependent system in which the addition of IPTG produced a dose-dependent increase in bioluminescence (Fig. 5). This experiment showed, therefore, that the intracellular expression of SP<sub>pelB</sub>-TgaA can cause the accumulation in *E. coli* cells of peptidoglycan breakdown products, possibly originating from cell wall lysis by TgaA.

Finally, purified SP<sub>pelB</sub>-TgaA displayed peptidoglycan lytic activity in a renaturing SDS-PAGE assay with cell wall substrates from *Micrococcus luteus* (see Fig. S3 in the supplemental material). We ran the same experiment also with the two TgaA protein domains (LT and CHAP), expressed separately by the preparation of recombinant forms of TgaA through overexpression in *E. coli*. Recombinant proteins lacked the original TgaA signal peptide and were named as follows: protein  $\Delta$ SP-TgaA- $\Delta$ CHAP-His (containing only the LT domain; rLT) and  $\Delta$ SP-TgaA- $\Delta$ LT-His (containing only the CHAP domain; rCHAP). Renaturing SDS-PAGE analyses clearly showed TgaA peptidoglycan lytic activity predominantly linked with the transglycosylase domain LT, as confirmed by a distinct band corresponding to the position to which rLT had migrated on SDS-PAGE (Fig. 6). In addition, the clarification determined by the rCHAP, although faint, was detectable (Fig. 6). These results agree with those of Arends et al., who found that both the LT and CHAP domains can degrade peptidoglycan (40).

In conclusion, our results show unambiguously that the TgaA protein displays peptidoglycan lytic enzymatic activity, which is consistent with the supposed VirB1-like role of this enzyme.

**Protein TgaA is expressed on the *B. bifidum* MIMBb75 cell surface.** The enzymatic activity of TgaA protein and its potential role in the putative T4SS suggest its location at cell wall level. Therefore, to confirm the presence and localization of the TgaA protein in *B. bifidum* cells, we prepared rabbit polyclonal antibodies against a purified recombinant form of TgaA protein. We first studied the presence of TgaA in MIMBb75 cells by fluorescence immunostaining with a goat anti-rabbit phycoerythrin (PE)-Cy5.5 secondary antibody via fluorescence microscopy, which revealed evident fluorescence emission by many cells, particularly by cell aggregates (Fig. 7A).

In addition, to directly pinpoint TgaA in *B. bifidum* MIMBb75 cells, we ran experiments using immunogold transmission electron microscopy. The specificity of TgaA recognition by the antibodies was demonstrated by the fact that cell staining was insignificant when we immunolabeled *B. bifidum* PRL2010, a bifidobacterial strain with no gene in its genome sharing significant sequence similarity with *tgaA*. In contrast, the electron micrographs showed numerous positive signals in the cytoplasm and, particularly, associated with the cell wall of MIMBb75 (Fig. 7B and C). Gold particles were also partially distributed in an extracellular matrix (Fig. 7B, frames c to f), which could indicate either a molecule naturally produced by the bacterium (e.g., exopolysaccharides) or cytoplasm extruded from bacterial cells during sectioning. These data confirm that the TgaA protein is abundantly expressed in *B. bifidum* MIMBb75. In particular,



experiments with intact cells established unequivocally that TgaA was present on the outer surface of the bacterial cells (Fig. 7B), consistent with the role proposed for VirB1-like proteins in T4SS (50).

**Conclusions.** The results of this study can be summarized as follows: (i) the genome of *Bifidobacterium bifidum* MIMBb75 contains a 45-kb region that includes 19 putative genes coding for a potential type IV secretion system; (ii) one gene of this region, named *tgaA*, putatively encodes the VirB1-like protein of the system, containing two enzymatic domains (lytic transglycosylase LT and amidase CHAP) reportedly associated with peptidoglycan lysis; (iii) we experimentally confirmed that protein TgaA, consistent with its putative VirB1 role, has peptidoglycan lytic activity and is abundantly expressed on the cell surface of *B. bifidum* MIMBb75.

T4SSs have not been characterized before in bifidobacteria. However, a database search revealed that a genetic region significantly similar to MIMBb75's putative T4SS is also present in the genome of *Bifidobacterium bifidum* BGN4 (93% nucleotide identity) and *Bifidobacterium longum* subsp. *infantis* 157F (91% nucleotide identity). In this study, we undertook the initial characterization of this genetic region, which, according to the literature, can have important implications for bacterial cell-to-cell communication as well as cross talk with host cells (19). In addition, the localization and abundance of TgaA on the *B. bifidum* cell surface support the hypothesis that this protein may potentially act as a molecular pattern recognized by host cell receptors at the intestinal level, justifying the interest for further studies aimed at the investigation of the direct interaction of TgaA protein with host cells. Accordingly, in a companion paper (32), we describe the ability of TgaA protein domains to activate dendritic cell maturation.

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