

Lactic Acid Bacteria as a Surface Display Platform for *Campylobacter jejuni* Antigens

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Key Words

Lactobacillus salivarius · *Lactococcus lactis* · GEM particles · *Campylobacter* · CjaA · CjaD · Vaccine

Abstract

Background: Food poisoning and diarrheal diseases continue to pose serious health care and socioeconomic problems worldwide. *Campylobacter* spp. is a very widespread cause of gastroenteritis. Over the past decade there has been increasing interest in the use of lactic acid bacteria (LAB) as mucosal delivery vehicles. They represent an attractive opportunity for vaccination in addition to vaccination with attenuated bacterial pathogens. **Methods:** We examined the binding ability of hybrid proteins to nontreated or trichloroacetic acid (TCA)-pretreated LAB cells by immunofluorescence and Western blot analysis. **Results:** In this study we evaluated the possibility of using GEM (Gram-positive enhancer matrix) particles of *Lactobacillus salivarius* as a binding platform for 2 conserved, immunodominant, extracytoplasmic *Campylobacter jejuni* proteins: CjaA and CjaD. We analyzed the binding ability of recombinant proteins that contain *C. jejuni* antigens (CjaA or CjaD) fused with the protein anchor (PA) of the *L. lactis* peptidoglycan hydrolase

AcmA, which comprises 3 LysM motifs and determines non-covalent binding to the cell wall peptidoglycan. Both fused proteins, i.e. 6HisxCjaAx3LysM and 6HisxCjaDx3LysM, were able to bind to nontreated or TCA-pretreated *L. salivarius* cells. **Conclusion:** Our results documented that the LysM-mediated binding system allows us to construct GEM particles that present 2 *C. jejuni* antigens. © 2015 S. Karger AG, Basel

Introduction

Campylobacter spp. is a leading cause of zoonotic enteric bacterial infections in most developed and developing nations. In developing countries, campylobacteriosis predominates as a pediatric disease, with an extremely high incidence rate (~40,000–60,000 per 100,000 population) in children under 5 years of age [Coker et al., 2002; Jones et al., 2007]. The incidence rate of campylobacteriosis is lower in developed countries such as the USA or European Union (EU) countries (20–100 per 100,000 population), occurring mainly as sporadic cases in young adults and infants [Jones et al., 2007]. The number of reported confirmed cases of human campylobac-

teriosis in the EU was 220,209 in 2011, which represented an increase of 2.2% compared to 2010. The EU notification rate was 50.28 per 100,000 population in 2011. However, as reported by the European Food Safety Authority (EFSA), there is considerable underascertainment and underreporting of the incidence of human campylobacteriosis; the true number of disease cases may range between 2 million and 20 million per year. The principal reservoirs of *Campylobacter* spp. are the alimentary tracts of wild and domesticated birds and mammals. There are multiple pathways for human exposure to the pathogen, including food, untreated drinking water, and contact with surface water or animals. Among these sources, broiler meat is unquestionably the main source of human infections due to the high levels of consumption by people. The occurrence of *Campylobacter* in broiler flocks in the EU in 2011 was high but varied widely among countries, ranging from 0 to 92.0% [EFSA, 2011; EFSA and ECDC, 2013]. According to the scientific opinion of the EFSA Panel on Biological Hazards, the handling, preparation, and consumption of undercooked broiler meat may account for 20–30% of human cases of campylobacteriosis, and 50–80% of human cases may be attributed to the chicken reservoir as a whole. It is estimated that reducing the numbers of *Campylobacter* in poultry intestines by 3 log₁₀ units at the time of slaughter would reduce the public health risk by at least 90%. Reducing the numbers of *Campylobacter* on the carcasses by 1 log₁₀ unit would reduce the public health risk by between 50 and 90% [EFSA, 2011]. Thus, the transmission of *Campylobacter* from poultry to humans is a serious public health concern. The prevention of human infections requires control measures at all stages of the food chain, from agricultural production on the farm to the processing, manufacture, and preparation of foods in both commercial establishments and the domestic environment. However, the main challenge is to develop an effective strategy to reduce the level of poultry stock infections. Given that the currently available biosecurity interventions appear to have limited effectiveness or are difficult to sustain, the most efficient strategy to decrease the number of human *Campylobacter* infections may be to implement an immunoprophylactic method, i.e. the protective vaccination of chickens. While it is known that maternal antibodies protect young chickens against *Campylobacter* colonization, there is no commercial vaccine against *Campylobacter* available to date [Cawthraw and Newell, 2010; Sahin, et al., 2003]. Due to the enormous genetic diversity observed among *C. jejuni*/*C. coli* strains, vaccination with live attenuated *Campylobacter*

strains or using killed, whole-cell *Campylobacter* appears to be ineffective. Additionally, an effective chicken vaccine should induce a rapid and strong immune response, yet due to the short life span of broiler chickens the birds should be immunized during the first week of life when the avian immune system is immature. A recent test of subunit vaccines that consisted of conserved *Campylobacter* antigens delivered by attenuated *Salmonella* strains, *Eimeria* or nanoparticles, generated promising results [Annamalai et al., 2013; Buckley et al., 2010; Clark et al., 2012; Layton et al., 2011; Łaniewski et al., 2012; Wyszynska et al., 2004]. Several detailed reviews recapitulating recent approaches to control *Campylobacter* in poultry via vaccination have been published within the last 5 years [Curtiss et al., 2007; de Zoete et al., 2007; Jagusztyn-Krynicka et al., 2009; Lin, 2009]. Lactic acid bacteria (LAB), which constitute a very heterogeneous group of Gram-positive, nonsporulating, low-GC-content microorganisms that are ubiquitous in many nutrient-rich environments, represent an attractive alternative for vaccinations that employ attenuated bacterial pathogens because their use eliminates the potential risk of increased virulence in young or immunocompromised individuals. Moreover, the mucosal administration of rLAB strains stimulates both systemic and mucosal immune responses against foreign antigens while only inducing a low-level immune response against the carrier strains [Bahey-El-Din, 2012; Bermudez-Humaran et al., 2011; Pontes et al., 2011; Wells and Mercenier, 2008]. In addition to using live LAB cells, nonliving trichloroacetic acid (TCA)-pretreated LAB cells (GEM particles – Gram-positive enhancer matrix) that are deprived of some surface components and intracellular content can also constitute a safe and efficient vaccine delivery vector for heterologous proteins [Audouy et al., 2007; Bosma et al., 2006; van Roosmalen et al., 2006]. There are 2 strategies used to present heterologous antigens on the surface of LAB cells. The first approach makes use of the C-termini of cell-anchoring proteins that contain the LPXTG recognition motif. This mechanism requires processing by a sortase for covalent anchoring of the protein to the cell wall peptidoglycan [Call and Klaenhammer, 2013; Schneewind and Missiakas, 2012]. The second approach is based on the protein anchor (PA) domain of some lactococcal proteins, such as AcmA. AcmA is the major autolysin of *Lactococcus lactis* that is required for cell separation and is responsible for cell lysis during the stationary phase of growth [Buist et al., 1997]. The autolysin contains 3 domains: the N-terminal signal sequence, an active site domain, and a C-terminal peptidoglycan-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Source or reference(s)
<i>Strains</i>		
<i>L. lactis</i> subsp. <i>lactis</i> IL1403	Plasmid-free strain	INRA; Chopin et al., 1984
<i>E. coli</i> TG1	<i>supE44 hsdΔ5 thiΔ (lac-proAB) F' [traD36 proAB⁺ lacI^q lacZΔM15]</i>	Sambrook and Russel, 2001
<i>E. coli</i> (DE3) BL21	<i>F⁻ ompT hsdSB (r_B⁻ m_B⁻) gal dcm</i> (DE3)	Novagen
<i>E. coli</i> Rosetta LysS (DE3)	<i>F⁻ ompT hsdSB (r_B⁻ m_B⁻) gal dcm</i> (DE3) pLysSRARE (Cam ^R)	Novagen
<i>L. salivarius</i> IBB3154	Isolated from chickens	This study
<i>C. jejuni</i> 81–176	Wild type; isolated from a child with bloody diarrhea during an outbreak in the USA; pVir, pTet (Tc ^R); Lior 5; Penner 23/26	Korlath et al., 1985
<i>Plasmids</i>		
pGEM-T Easy	Ap ^R ; T vector for cloning PCR products	Promega
pET28	Km ^R ; lacI; overexpression vector	Novagen
pUWM1146	<i>pelB_{SS}-cjaA-6xhis</i> fusion in pET22b	Łaniewski et al., 2014
pUWM1288	<i>cjaA-lysM</i> fusion in pGEM-T Easy	This study
pUWM1313	<i>6xhis-cjaA-lysM</i> fusion in pET28a	This study
pUWM1290	<i>cjaD</i> in pGEM-T Easy	This study
pUWM1292	<i>6xhis-cjaD</i> fusion in pET28a	This study
pUWM1287	<i>cjaD-lysM</i> fusion in pGEM-T Easy	This study
pUWM1282	<i>6xhis-cjaD-lysM</i> fusion in pET28a	This study

binding domain (PA). The PA comprises 3 LysM motifs, each with about 45 amino acids, separated by spacer sequences. After secretion, AcmA is directed to the cell wall and its C-terminus determines its noncovalent binding to the cell wall peptidoglycan [Buist et al., 2008]. It has been demonstrated that hybrid PA fusions exhibit similar properties [Bosma et al., 2006; Raha et al., 2005; Steen et al., 2003]. Also LysM domains of other Gram-positive bacteria bind heterologous proteins to the cell wall peptidoglycan [Hu et al., 2010; Turner et al., 2004; Xu et al., 2011].

This work consists of an attempt to evaluate the possibility of using GEM particles of *Lactobacillus salivarius* as a binding platform for 2 conserved, immunodominant, extracytoplasmic *Campylobacter* proteins: CjaA (Cj0982c in the genome of *C. jejuni* NCTC11168) and CjaD (Cj0113 in the genome of *C. jejuni* NCTC11168). Both proteins have been intensively studied as chicken vaccine candidates [Buckley et al., 2010; Clark et al., 2012; Layton et al., 2011; Łaniewski et al., 2012; Wyszynska et al., 2004].

Materials and Methods

Bacterial Strains, Primers, Plasmids, Media, and Growth Conditions

The bacterial strains, plasmids, and primers used in this study are listed in tables 1 and 2. *L. lactis* IL1403 and *L. salivarius* IBB3154

were used in this study. The *L. salivarius* IBB3154 belongs to the Regional Strains and Plasmids Collection of the IBB PAS (Institute of Biochemistry and Biophysics, Polish Academy of Sciences). The *L. lactis* IL1403 strain was routinely cultured at 30°C in M17 broth (Oxoid) containing 0.5% (weight/volume) glucose (GM17). The *L. salivarius* strain was cultured in MRS liquid or MRS agar (solidified with 1.5% agar) medium (Difco Laboratories, Detroit, Mich., USA) at 37°C. The *E. coli* strain TG1 was used as a host for the construction of recombinant plasmids. The *E. coli* strain Rosetta (DE3) LysS was used to overexpress pUWM1320 and pUWM1292, and the *E. coli* strain BL21 (DE3) was used to overexpress pUWM1282 and pUWM1146 [Łaniewski et al., 2014]. *C. jejuni* and *E. coli* strains were grown under standard conditions [Łaniewski et al., 2014] unless otherwise indicated. When needed, media were supplemented with antibiotics at the following concentrations: 100 μg·ml⁻¹ ampicillin, 30 μg·ml⁻¹ kanamycin, and 20 μg·ml⁻¹ chloramphenicol, as well as *Campylobacter* Selective Supplement (Oxoid) and IPTG (3 mg·ml⁻¹) in DMF (dimethylformamide).

General DNA Manipulations

Standard DNA manipulations were carried out as described earlier by Sambrook and Russel [2001] or according to the manufacturer's instructions (A&A Biotechnology, Poland). The chromosomal DNA of *C. jejuni* 81–176 and *L. lactis* used for PCR reactions were isolated using a commercial kit and protocol (A&A Biotechnology). PCR were performed with PrimeStar HS DNA Polymerase (TaKaRa) or HotStar HiFidelity Polymerase (Qiagen) under standard conditions. Synthetic oligonucleotide synthesis and DNA sequencing for cloning experiments were performed by Genomed S.A., Warsaw, Poland. DNA sequencing of the PCR product spanning the 16S rDNA gene was performed by the DNA Sequencing and Oligonucleotide Synthesis Service at IBB PAS in

Table 2. Oligonucleotides used in this study

Name	Sequence (5'→3')	Orientation/restriction site
Cj0148NheI	ACTGCGCTAGCTGTAGCACAAAAAGCACTAGC	Forward/NheI
Cj0148LysM	GAAGAAGCACCGTCAAAAACGAGTCTTGATAATTTAAATTCAG	Reverse/Ø
Cj0148RXhoI	GCCTCGAGTTATCTTGATAATTTAAATTCAG	Reverse/XhoI
Cj0148BamHI	CCGGATCCTGTAGCACAAAAAGCACTAGC	Forward/BamHI
LysMF	ACTCGTTTTGACGGTGCTTCTTC	Forward/Ø
LysMRXho	ACCTCGAGTTATTTAATACGAAGATATTG	Reverse/XhoI
CjaANheI	ACTGCGCTAGCGGAGGAAATTTCTGACTC	Forward/NheI
CjaALysM	GCACCGTCAAAAACGAGTAATTTTTCCACCTTCAATCAC	Reverse/Ø
16-1A	GAATCGCTAGTAATCG	Forward/Ø
23-1B	GGGTTCCCCATTCGGA	Reverse/Ø
343F	TACGGGAGGCAGCAG	Forward/Ø
1406R	ACGGGCGGTGTGTRC	Reverse/Ø

Restriction enzyme recognition sites introduced for cloning purposes are underlined. All primers were designed on the basis of the *C. jejuni* 81–176 and *L. lactis* IL1403 genome nucleotide sequences. Bold letters indicate nucleotides complementary to the *C. jejuni* 81–176 chromosome nucleotide sequence. The oligos in italics were designed for overlap PCR to construct the *cjaA-lysM* and *cjaD-lysM* fusion genes.

Warsaw, using the sequencer ABI377 (Applied Biosystems), and then the nucleotide sequences were analyzed using BLAST against the nucleotide database on the NCBI website.

Construction of Recombinant Plasmids for Recombinant Protein Overexpression

A *CjaD* expression vector was made as follows. The *cjaD* coding sequence (without the signal sequence, amino acids 1–18) was amplified from *C. jejuni* 81–176 chromosomal DNA with the primers Cj0148BamHI and Cj0148RXhoI and cloned into pGEM-T Easy (Promega, Madison, Wisc., USA). Thereafter, the resulting plasmid, i.e. pUWM1290, was digested with XhoI and BamHI restriction enzymes and a 0.5-kb DNA fragment was inserted into pET28a, generating plasmid pUWM1292. The construction of the *CjaA* expression vector (pUWM1146) has been described previously [Łaniewski et al., 2014].

The *CjaA-LysM* (pUWM1320) and *CjaD-LysM* (pUWM1282) expression vectors were constructed via a 2-step PCR method. Briefly, the primers LysMF and LysMRXhoI were used to amplify the DNA region encoding the C-terminal portion of Acma (amino acids 211–439) from the chromosome of *L. lactis* IL1403. CjaANheI-CjaALysM and Cj0148Nhe-Cj0148LysM were used to amplify the *cjaA* and *cjaD* genes, respectively, from the chromosome of *C. jejuni* 81–176. The CjaALysM and Cj0148LysM primers contained 5' leader nucleotide sequences complementary to LysMF. Each PCR product was purified using a Gel-Out extraction kit (A&A Biotechnology). Next, a mixture of 2 purified products (in equal amounts) was used as a template in a single PCR reaction, using the primers CjaANheI-LysMF or Cj0148Nhe-LysMF to amplify *cjaA-lysM* and *cjaD-lysM*, respectively. The resulting PCR products were purified and cloned into pGEM-T Easy to generate pUWM1288 and pUWM1277, respectively. Finally, using XhoI and NheI restriction enzymes, the 1.5-kb DNA region encoding *CjaALysM* and the 1.1-kb DNA fragment encoding *CjaDLysM* were transferred into pET28b in the native orientation, generating

pUWM1320 and pUWM1282, respectively. Correct construction of the plasmids was verified by sequencing. Protein production was confirmed by Western blot using a previously obtained rabbit polyclonal anti-rCjaD or anti-rCjaA serum [Łaniewski et al., 2012; Pawelec et al., 2000]. All recombinant plasmids encode proteins with a 6His tag fused to their N-terminus to allow purification by affinity chromatography.

Overexpression and Purification of *CjaA*, *CjaD*, *CjaALysM*, and *CjaDLysM*

CjaDLysM and *CjaA* were overexpressed and purified from *E. coli* BL21 harboring pUWM1282 and pUWM1146, respectively. *CjaD* and *CjaALysM* were overexpressed and purified from *E. coli* Rosetta (DE3) LysS harboring pUWM1292 and pUWM1320, respectively. Expression of *CjaD* and *CjaDLysM* was induced with 0.5 mM IPTG at OD_{600 nm} of ~0.6 from cells growing at 18°C. *CjaA* and *CjaALysM* were overexpressed by autoinduction as described by Studier [2005]. After 24 h, cultures were centrifuged and cell pellets were suspended in 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 10 mM imidazole. Cells were disrupted by sonication. Subsequently, the cell lysates were centrifuged and the resulting supernatants were applied onto a HisTrap column (Novagen). The proteins were eluted with an imidazole gradient. Fractions containing *CjaD*, *CjaA*, *CjaDLysM*, or *CjaALysM* were pooled and loaded onto a PD-10 column (GE Healthcare) equilibrated with 50 mM sodium phosphate (pH 8.0) and 150 mM NaCl. Overexpression and all purification steps were monitored via SDS-PAGE.

SDS-PAGE and Western Blotting

SDS-PAGE and Western blotting procedures were done using standard techniques. Blots were developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) as a substrate using previously obtained rabbit polyclonal anti-rCjaA or anti-rCjaD serum [Łaniewski et al., 2012; Pawelec et

al., 2000] or anti-His antibodies (Sigma-Aldrich) as primary antibodies and mouse anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) or goat anti-mouse IgG alkaline phosphatase conjugate as secondary antibodies.

Preparation of Live Cells and GEM Particles for the Binding Assay

Live cells for the binding assay were prepared from 1-ml cultures (*L. salivarius* IBB3154 and *L. lactis* IL1403) with an absorbance of about 1.0 at OD_{600 nm}. Cells were collected by centrifugation and washed once with a 0.5 volume of phosphate-buffered saline (PBS) (58 mM Na₂HPO₄, 2H₂O, 17 mM Na₂H₂PO₄, H₂O, 68 mM NaCl, pH 7.2). GEM particles were obtained via chemical pretreatment with 10% TCA (0.6 M). This method is commonly used for peptidoglycan purification from cell wall extracts. Briefly, bacterial cells from 1-ml cultures (*L. salivarius* IBB3154 and *L. lactis* IL1403) with an absorbance of about 1.0 at OD_{600 nm} were collected by centrifugation and washed once with a 0.5 volume of PBS. Next, the washed cells were resuspended in 200 µl of a 10% TCA solution and boiled for 30 min [Bosma et al., 2006]. Then, the GEM particles were washed 3 times with PBS. For protein binding, the prepared live cells and GEM particles were resuspended in MRS mixed with 1,000 pmol of recombinant protein (CjaA, CjaD, CjaALysM, or CjaDLysM) and incubated 60 min. Binding of the proteins to live LAB cells or GEM particles was analyzed by immunofluorescence and Western blot assays.

Binding Assays by Immunofluorescence and Western Blot Analysis

For the immunofluorescence assay, *L. salivarius* IBB3154 and *L. lactis* IL1403 cells and GEM particles that were mixed with protein were washed 3 times with 1× PBS and then resuspended in 4% (weight/volume) paraformaldehyde and incubated for 10 min at room temperature. Next, cells were washed with 1× PBS and resuspended in PBS containing 3% bovine serum albumin. After 30 min of incubation, primary rabbit anti-rCjaA or anti-rCjaD antibodies were added at a dilution of 1:100, followed by additional incubation for 60 min at room temperature. Then the cells were washed 3 times with 1× PBS and resuspended in PBS containing 1% Tween 20. The cells were washed 3 more times with 1× PBS and then incubated for 1 h at room temperature with 100 µl of PBS containing 3% bovine serum albumin and the secondary anti-rabbit antibody Alexa fluor A488 diluted 1:100. Next, the cells were washed 3 times with 1× PBS and resuspended in 50 µl of PBS. A 10-µl aliquot of the suspension was spread onto a chamber slide precoated with poly-L-lysine. This was followed by mounting in an antifading agent (fluorescence mounting medium; Dako). Fluorescence was visualized using a Nikon A1R MP microscope.

For the Western blot assay, GEM particles made from *L. salivarius* or live cells of *L. salivarius* were incubated with recombinant proteins for 1 h under gentle agitation at 37°C. Subsequently, they were centrifuged and the supernatants were retained for analysis. Next, the cells with bound proteins were washed 3 times with PBS and then resuspended in PBS for analysis. The supernatant and the cells were mixed with 4× SDS loading-dye and boiled. The proteins derived from equal amounts of culture extracts were analyzed by Western blot using polyclonal anti-rCjaA or anti-rCjaD serum [Łaniewski et al., 2012; Pawelec et al., 2000].

Results and Discussion

Characterization of *L. salivarius* Strains

The aim of this work was to generate an LAB surface display system for *C. jejuni* antigens. We used 2 LAB strains: *L. lactis*, the LAB model microorganism tested as a mucosal delivery vector for therapeutic proteins and vaccines, and *L. salivarius*. The *Lactobacillus* genus has served as a delivery vector of heterologous antigens for mucosal immunization, and the genus has added advantages over *Lactococcus*: lactobacilli can persist longer than lactococci in the digestive tract and some strains have probiotic properties [Turner et al., 2004]. The *L. salivarius* IBB3154 strain was isolated from chicken stool samples on MRS agar medium. Macroscopic and microscopic (see online suppl. fig. S1; for all online suppl. material, see www.kager.com/doi/10.1159/000368780) observations suggested that this isolated strain belonged to the genus *Lactobacillus*. The strain grew as white colonies on MRS agar medium (online suppl. fig. S1A) and formed short rods that could be clearly seen by phase contrast microscopy (online suppl. fig. S1B). The morphological and microscopic observations were confirmed by molecular identification: amplification of an intergenic chromosomal DNA region between the 16S rDNA and 23S rDNA using the genus-specific primer pairs 16-1A and 23-1B [Tannock et al., 1999]. This approach generated 2 polymerase chain reaction (PCR) products of 500 and 700 bp (online suppl. fig. S2) which are characteristic of strains belonging to the genus *Lactobacillus*. This indicated that the strain belonged to the genus *Lactobacillus*. To identify the species of the IBB 3154 strain, the 16S rDNA gene was amplified using 343F and 1406 primers (online suppl. fig. S3). A PCR product of the expected size, i.e. 1 kb, was generated in amounts sufficient for DNA sequencing using the same 2 primers. The nucleotide sequence of this 16S rDNA PCR product was analyzed using BLAST against the nucleotide database on the NCBI website. The analysis revealed the highest identity, i.e. 99%, to the nucleotide sequence of the 16S rDNA gene of *L. salivarius* IBB3154 (GeneBank CP002034.1), thus identifying the taxonomic position of the strain as *L. salivarius* IBB3154. Because this *L. salivarius* IBB3154 strain was isolated from a chicken's digestive track, we speculate that it is able to colonize the chicken intestine. Using live *Lactobacillus* might facilitate vaccination procedures and lower the cost of immunization. However, because the efficacy of immunization is often dependent on, among others factors, the amount of antigen deliv-

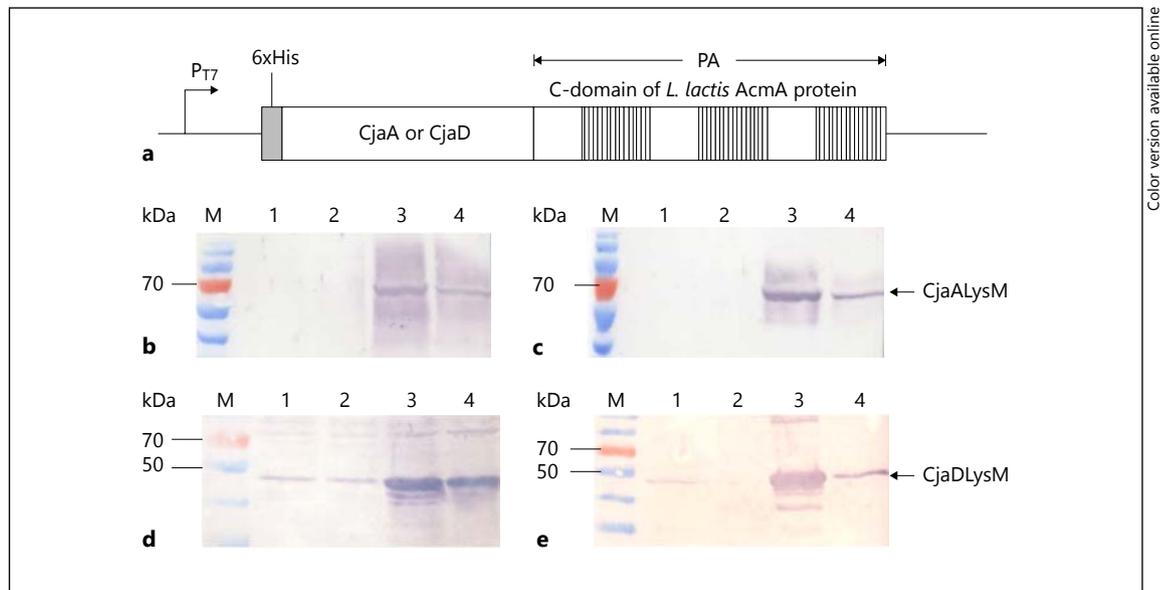


Fig. 1. Construction and purification of the hybrid proteins which consist of *Campylobacter* antigens fused to the C-domain of *L. lactis* AcmA. **a** Schematic diagram of the genes encoding the hybrid proteins 6HisxCjaAx3LysM or 6HisxCjaDx3LysM. Hatching boxes indicate the LysM repeat sequences. **b, c** Western blot analysis of the CjaALysM produced in the *E. coli* Rosetta (DE3) LysS strain by autoinduction. **d, e** Western blot analysis of the CjaDLysM produced in the *E. coli* (DE3) BL21 strain by IPTG induction. The arrows indicate the position of the CjaALysM (**b, c**) or CjaDLysM

(**d, e**) fused proteins. The protein extracts from collected fractions (equal volumes of fractions were loaded per lane) were separated using 12% SDS-PAGE under reducing conditions and probed with: anti-CjaA antibodies (**b**), anti-CjaD antibodies (**d**), or anti-His antibodies (**c, e**). Lanes: 1 – cell extract before induction, insoluble fraction; 2 – cell extract before induction, soluble fraction; 3 – cell extract after 24 h of induction, insoluble fraction, 4 – cell extract after 24 h of induction, soluble fraction, and M – protein molecular-weight marker.

ered, GEM particles might be more effective than live cells. Thus, in this work we evaluated the possibility of employing *L. salivarius* GEM particles as a platform to present *C. jejuni* antigens.

C. jejuni Antigens Bind to LAB Cells via the LysM-Mediated Surface Display System

A crucial factor that determines the efficacy of subunit vaccines is the selection of antigen(s). As many *Campylobacter* strains can colonize the chicken at one time and because of the enormous genetic diversity observed among *Campylobacter* isolates, the potential vaccine candidate protein should be conserved among different pathogen serotypes/genotypes to induce cross-protection. To evaluate the potential of *L. salivarius* GEM particles as a delivery vector for *C. jejuni* antigens, we chose 2 immunodominant proteins: CjaA (Cj0982c in the genome of *C. jejuni* NCTC11168) and CjaD (Cj0113 in the genome of *C. jejuni* NCTC11168). CjaD is a peptidoglycan-associated protein (PAL) anchored in the outer membrane [Godlewska et al., 2009]. CjaA is the cysteine-binding protein components of the ABC transport system [Muller et al.,

2005; Wyszynska et al., 2004, 2008]. It participates in the in vivo colonization process as the expression levels of the *cjaA* gene increase when bacterial cultures are grown in media that is deficient in iron or on solid media [Holmes et al., 2005]. Additionally, CjaA is more abundant in the proteome of clinical *Campylobacter* isolates compared to the proteome of the laboratory strain, and it is recognized by chicken maternal antibodies [Cordwell et al., 2008; Shoaf-Sweeney et al., 2008]. We analyzed the LAB cell wall-binding ability of recombinant proteins containing *C. jejuni* antigens (CjaA or CjaD) that were fused with the PA binding domain of the *L. lactis* peptidoglycan hydrolase AcmA, which contains 3 lysine motifs (LysM). For this purpose, 2 recombinant plasmids expressing fused proteins were constructed (fig. 1a). To facilitate purification of the recombinant proteins, the hybrid proteins also contained a 6His tag at their N-terminus. The fused proteins 6HisxCjaAx3LysM (hereinafter referred to as CjaALysM) and 6HisxCjaDx3LysM (hereinafter referred to as CjaDLysM) were produced using an *Escherichia coli* expression system and purified by affinity chromatography. Western blot analysis with specific rabbit anti-CjaA/

anti-CjaD and anti-His antibodies confirmed their specificity (fig. 1b–e). Both hybrid proteins were tested for the ability to bind to nontreated or TCA-pretreated *L. salivarius* cells. CjaA and CjaD proteins, which were also obtained using an *E. coli* expression system and purified by affinity chromatography, were employed as negative controls in these assays. The binding was examined by confocal microscopy assay using specific anti-CjaA or anti-CjaD rabbit antibodies and a secondary fluorescent donkey anti-rabbit IgG conjugated with Alexa fluor 488. We examined the ability of hybrid proteins to bind to an *L. salivarius* IBB3154 strain isolated from a chicken intestinal tract. *L. lactis* IL1403 was used as a control. The results are given in figure 2 and online supplementary figure S4 (control experiments). We found that the binding efficiency was greater for TCA-pretreated cells (GEM particles) than for live, untreated cells regardless of the strains used. Binding to untreated cells was restricted to specific locations on the cell surface, which is consistent with data published by others [Bosma et al., 2006; Hu et al., 2010], while the hybrid proteins were able to bind to the entire surface of the GEM particles. Control experiments lacking any added proteins or employing nonfused CjaA or CjaD proteins documented the specificity of the used sera and showed that generally binding is LysM dependent (online suppl. fig. S4) (more than 90% of the cells showed staining). As the CjaD protein is a peptidoglycan-associated protein, it also binds to peptidoglycan. In the case of GEM particles, binding was also observed for nonfused CjaA protein. However the process is not as intense as in the case of CjaALysM protein.

The LysM-Mediated Binding System Allows the Construction of GEM Particles that Present Two C. jejuni Antigens

To shed more light on the binding process and to clearly define whether the binding capability is dependent on the structure of the *Campylobacter* antigen, we decided to evaluate the binding efficiency of the recombinant proteins by Western blot. As GEM particles bound more protein than live cells, we mainly concentrated on experiments employing GEM particles. GEM particles were separated from the supernatant by centrifugation and the amount of hybrid proteins on the surface of the GEM particles and in the supernatant was determined. Control samples contained CjaA or CjaD protein. The results are given in figure 3. This experiment confirmed that hybrid proteins are able to bind to GEM particles, whereas control proteins (CjaA or CjaD) that lack the LysM domain remain free in the supernatant, especially CjaA (fig. 3a,

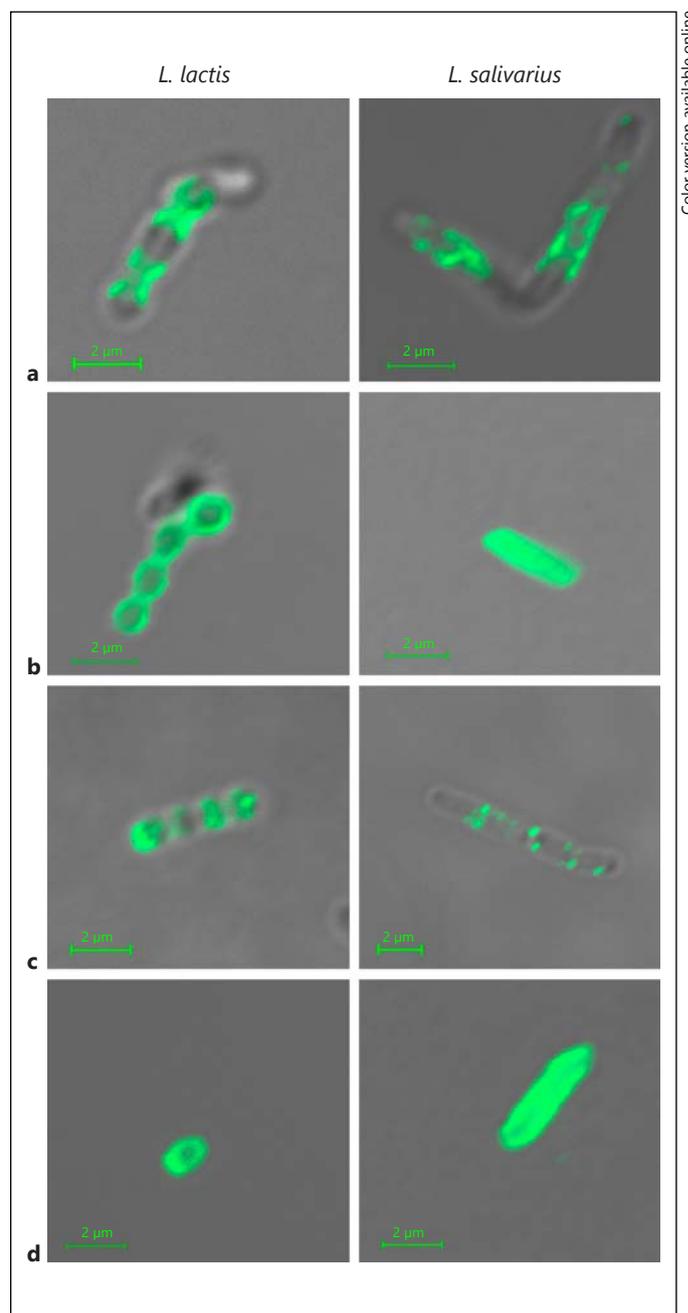


Fig. 2. Localization of CjaALysM and CjaDLysM on *L. lactis* and *L. salivarius* cells. **a** Localization of the CjaALysM fusion protein on nonpretreated *L. lactis* and *L. salivarius* cells. **b** Localization of the CjaALysM fusion protein on TCA-pretreated *L. lactis* and *L. salivarius* cells. **c** Localization of the CjaDLysM fusion protein on nonpretreated *L. lactis* and *L. salivarius* cells. **d** Localization of the CjaDLysM fusion protein on TCA-pretreated *L. lactis* and *L. salivarius* cells. Scale bar = 2 μm. The data generated from control experiments are given in online suppl. figure S4.

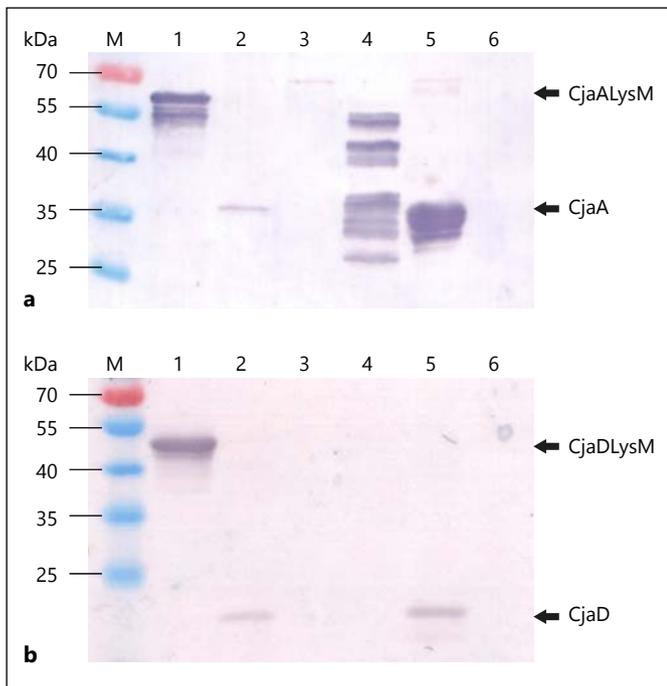


Fig. 3. Analysis of CjaALysM and CjaA (a) and CjaDLysM and CjaD (b) binding to GEM particles by Western blot using polyclonal anti-CjaA or anti-CjaD rabbit antibodies and anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) antibodies. GEM were mixed with chimeric proteins (CjaALysM or CjaDLysM) or with proteins lacking the LysM domain (CjaA or CjaD). Samples were prepared by boiling in 4× SDS loading dye. Proteins were resolved by 12% SDS-PAGE, transferred onto nitrocellulose, and probed with antibodies. Lanes 1–3 contain equal amounts of GEM particles mixed with: 1 – fusion protein; 2 – protein without LysM, and 3 – control, no recombinant protein. Lanes 4–6 contain equal amounts of supernatant fractions obtained after removing GEM particles by centrifugation. GEM mixed with: 4 – fusion protein; 5 – protein without LysM; 6 – control, no recombinant protein, and M – protein molecular-weight marker.

lane 5). This effect is less pronounced for CjaD. Probably CjaD, a peptidoglycan-binding protein, can also stimulate binding to GEM particles. The result is consistent with the data obtained by confocal microscopy assay. The strong binding efficiency of CjaDLysM was also observed with the *L. salivarius* live strain (online suppl. fig. S5). We also found that the CjaALysM present in the supernatant was subject to extensive degradation (fig. 3a, lane 4). We concluded that the process is stimulated by the PA domain of the fused protein, as it is not correctly localized.

It is generally accepted that successful chicken immunization against *Campylobacter* colonization should consist of several protective antigens or their epitopes delivered by an appropriate vector. Thus we decided to deter-

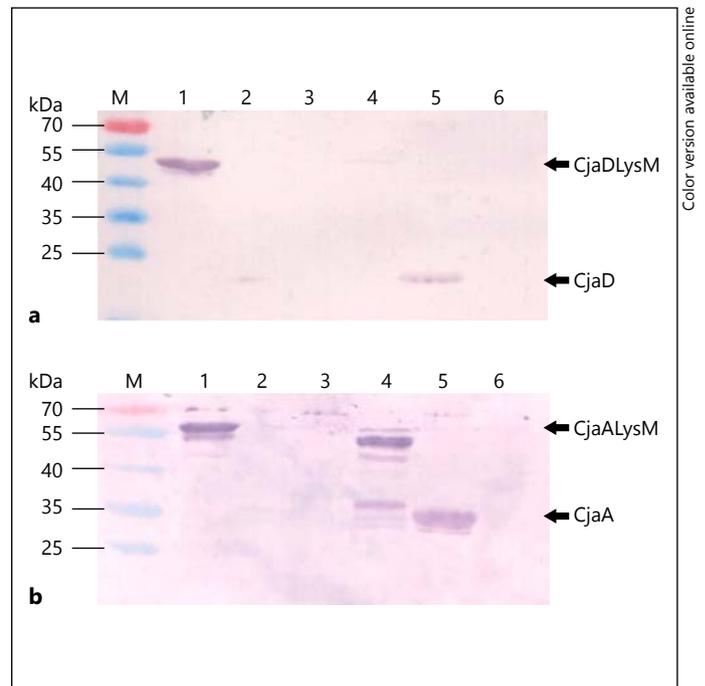


Fig. 4. Simultaneous binding of CjaALysM and CjaDLysM to GEM particles determined by Western-blot with anti-CjaD (a) and anti-CjaA rabbit antibodies (b). Recombinant CjaA and CjaD that are not fused to LysM were used as a control. GEM were mixed with CjaALysM and CjaDLysM or with CjaA and CjaD. Samples were prepared by boiling in 4× SDS loading dye. Proteins were resolved by 12% SDS-PAGE, transferred onto nitrocellulose, and probed with antibodies. Lanes 1–3 contain equal amounts of GEM particles mixed with: 1 – CjaALysM and CjaDLysM; 2 – CjaA and CjaD, and 3 – control, no recombinant protein. Lanes 4–6 contain equal amounts of supernatant fractions obtained after centrifugation of GEM particles mixed with: 4 – CjaALysM and CjaDLysM; 5 – CjaA and CjaD; 6 – control, no recombinant protein, and M – protein molecular-weight marker.

mine whether GEM particles can be used as a platform to present both the CjaA and CjaD antigens together as a potential divalent vaccine. We suspected that CjaDLysM, having a stronger ability to bind to cell wall peptidoglycan than CjaALysM, might remove CjaALysM from the GEM surface. To analyze the problem, GEM particles were reacted with the 2 purified hybrid proteins mixed in equal amounts. After binding, GEM particles were collected by centrifugation and washed, and the binding efficiency was determined by SDS-PAGE and immunoblotting. The results are given in figure 4. We found that although a noticeable amount of CjaALysM stayed in the supernatant, a significant amount of the fusion was also bound to the GEM surface. Thus we concluded that even though

CjaDLysM has a higher binding efficiency than CjaALysM it does not block CjaALysM binding.

In conclusion, this study demonstrates the potential use of LAB GEM particles to deliver *Campylobacter* antigens. Our results clearly indicate that GEM can be used as delivery vehicles for more than one antigen, and GEM may represent a good mucosal delivery tool for future applications in different chicken diseases.

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