

Identification and phenotypic characterization of the cell-division protein CdpA

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Abstract

Analysis of the automated computer annotation of the early draft phase genome of *Lactobacillus acidophilus* NCFM revealed the previously discovered S-layer gene *slpA* and an additional partial ORF with weak similarities to S-layer proteins. The entire gene was sequenced to reveal a 1799-bp gene coding for 599 amino acids with a calculated molecular mass of 64.8 kDa. No transcription or translation signals could be determined in close proximity to the 5' -region. However, a strong putative terminator with a free energy of -16.84 kcal/mol was identified directly downstream of the gene. A PSI-Blast analysis showed similarities to members of S-layer proteins, cell-wall associated proteinases and hexosyl-transferases. Calculation of an unrooted phylogenetic tree with other examples of S-layer proteins and proteinases placed the deduced protein separately from both groups. A derivative of *L. acidophilus* NCFM was constructed by targeted integration into the gene. SDS-PAGE analysis of non-covalently linked proteins of the cell wall of the mutant, compared to the wild type, revealed the loss of a cell-surface protein. Phenotypic analyses of the mutant revealed significant changes in cell morphology, altered responses to various environmental stresses, and lowered cell adhesion. Based on the in silico and functional analyses, we ascertained that this protein plays a role in cell-wall processing during the growth and cell-cell separation and designated the gene as cell-division protein, *cdpA*.

1. Introduction

Lactic acid bacteria have been used for centuries in fermentation processes and recently are gaining increased attention due to their probiotic properties. Among others,

these properties include reduced risk and reoccurrence of certain types of cancer, pathogen exclusion and immune modulation (Ouwehand et al., 2002). The ability to survive passage through the intestinal tract and potentially establish residence there are considered important features. The degree of retention is likely dependent on the ability of the bacteria to interact with eukaryotic cell surfaces or with the mucosal layer surrounding these cells. External structures of bacterial cells such as S-layer (Hynonen et al., 2002), fibronectin- (Christie et al., 2002; Kushiro et al., 2001) and mucin-binding proteins (Jonsson et al., 2001), may play a role in bacterial cell adhesion. S-layers are a common feature among bacteria and are found within all major phylogenetic groups, including the archaea (Sara and Sleytr, 2000). This indicates that S-layers provide a significant advantage in varied habitats, but these advan-

Abbreviations: aa, amino acids; bp, basepairs; *cdpA*, gene encoding for cell-division protein A; CdpA, cell-division protein A; cfu, colony forming units; Cm, chloramphenicol; CSDP, 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3,3,1.1^{3,7}]decan}-4-yl) phenyl phosphate; Er, erythromycin; GIT, gastrointestinal tract; gp, geneproduct; kDa, kilodalton(s); Km, kanamycin; LB, Luria-Bertani; MRS, deMan Rogosa Sharpe; nt, nucleotide(s); OD, optical density; ORF, open reading frame; PAGE, PA-gel electrophoresis; SDS, sodium dodecyl sulfate.

tages have not been clearly resolved. In gram-positive bacteria, S-layers are linked to the peptidoglycan-containing layer or the pseudomurein (Sara and Sleytr, 2000). S-layers found on the surface of some lactobacilli could interact with components of the epithelial cell layer of the mammalian gastrointestinal tract and have been proposed to affect adherence (Lorca et al., 2002), cell survival properties, and competitive inhibition of pathogenic bacteria adhering to damaged intestinal tissue (Horie et al., 2002). Other applications include the display of foreign epitopes fused to S-layer proteins (Avall-Jaaskelainen et al., 2002; Ilk et al., 2002).

We are investigating mechanisms underlying the probiotic properties of *Lactobacillus acidophilus* NCFM and applying genetic approaches to improve stability and functionality. In this study, we describe a newly discovered protein and investigated its role in cell division and morphology.

2. Material and methods

2.1. Bacterial strains and growths conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown aerobically in Luria–Bertani (LB) medium or on LB medium supplemented with 1.5% agar at 37 °C. *L. acidophilus* strains were propagated anaerobically in MRS broth (Difco, Detroit, MI) or MRS broth supplemented with 1.5% agar at 37 °C. Kanamycin (Km, 40 µg/ml), erythromycin (Er, 5 µg/ml and 150 µg/ml) and chloramphenicol (Cm, 5 µg/ml) were added where appropriate. Environ-

mental stress responses in *L. acidophilus* strains were actuated by supplementing the growth or recovery media with different concentrations of NaCl (w/v), Oxgall (w/v) and ethanol (EtOH) (v/v).

2.2. DNA isolation, manipulations, and transformations

Lactobacillus genomic DNA was isolated according to the method of Walker and Klaenhammer (1994). DNA isolations and manipulations in this study were performed according to standard procedures (Sambrook et al., 1989). Large-scale plasmid preparations were performed with the QIAprep Spin Midiprep kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Calf intestinal phosphatase and T4 DNA polymerase were purchased from New England Biolabs (Beverly, MA). All enzymes were used according to the manufacturers' specifications. DNA fragments were purified from agarose gels using the QIAEX II gel extraction kit (QIAGEN). All PCRs were performed according to the manufacturer's recommendations using either *Taq*- or the *Pwo* DNA polymerase PCR system (Roche Molecular Biochemicals). PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). When appropriate, restriction sites were designed into the 5' end of the primers to facilitate cloning. PCR products were purified using the QIAquick PCR purification kit (Qiagen).

Electrocompetent *E. coli* EC1000 cells (Law et al., 1995) were prepared and transformed as described by Dower et al. (1992) with a gene pulser (Bio-Rad Laboratories, Hercules, CA). Electrocompetent *Lactobacillus* cells were prepared using 3.5× SMEB as described by Luchansky et al. (1988).

Table 1
Bacterial strains, plasmids and amplification oligonucleotides

	Origin or relevant characteristics	Source or reference
<i>Strains</i>		
<i>L. acidophilus</i>		
NCFM	Human intestinal isolate	Barefoot and Klaenhammer, 1983
NCK1375	NCFM <i>cdp</i> ::pTRK828	This study
<i>E. coli</i>		
EC1000	RepA ⁺ MC1000, Km ^r , carrying a single copy of the pWV01 <i>repA</i> gene in the <i>glgB</i> gene; host for pORI28-based plasmids	Law et al., 1995
<i>Plasmids</i>		
pORI28	Er ^r , ori (pWV01), replicates only with <i>repA</i> provided in <i>trans</i>	Law et al., 1995
pTRK669	ori (pWV01), Cm ^r , RepA ⁺	Russell and Klaenhammer, 2001
pTRK828	2.4 kb; pORI28 with 777-bp internal <i>L. acidophilus</i> NCFM <i>cdpA</i> fragment	This study
<i>Oligonucleotides</i>		
Sequence		
cdp-A	5' -CGGAATTCTGCTTCAGCTTCATCAACTACT-3'	This study
cdp-B	5' -CGGAATTCTTCTCCATCTGGTTCATTGATA-3'	This study
cdp-I	5' -TCAGGTGCTAAGCTTGATCCTATCAAT-3'	This study
cdp-II	5' -ACGTATGAATTACGAGCAATCTTAATG-3'	This study
cdp-III	5' -TGCTTCACAACCTATTGCTCAAGTTA-3'	This study
cdp-IV	5' -AGCTGCTGGATACCATAATCAACT-3'	This study
cdp-V	5' -TAACTTGAGCAATAGTTGTGAAGCA-3'	This study
cdp-VI	5' -AAGTTGATTATGGTATCCAGCAGCT-3'	This study

2.3. DNA sequencing and analysis

DNA sequencing was performed at the University of California-Davis Automated DNA Sequencing Facility on an ABI Prism 377 DNA sequencer with a 96-lane upgrade (Applied Biosystems, Foster City, CA). Assembly and analysis of DNA sequences were performed with Clone Manager 5 (Scientific and Educational Software, Durham, NC). Protein homology and conserved motif searches were performed with the Basic Local Alignment Search Tool (BLAST), version 2.2.4, at the website of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), protein motifs were identified by comparison to the PROSITE (<http://www.expasy.ch/prosite/>) and PFam (<http://pfam.wustl.edu/hmmsearch.shtml>) databases. Amino acid alignments were performed with ClustalX (<http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html>) and phylogenetic trees were generated from ClustalX output by MEGA 2 (Molecular Evolutionary Genetics Analysis) (<http://www.megasoftware.net/text/overview.sht>). Automated annotation of the early draft phase genome of *L. acidophilus* NCFM was realized using the annotation suite GAMOLA (Altermann and Klaenhammer, 2003). Keyword-based data mining of the annotation results was done using “LightningSearch 1.03” (<http://www.uksiland.com/projects.html>).

2.4. Determination of the complete DNA sequence of *cdpA*

Restriction analysis of the DNA sequence of contig428 revealed no recognition site for *Bgl*III within the partial *cdpA* gene. Genomic DNA was restricted with *Bgl*III and subsequently recircularized. Using primers *cdp*-I and *cdp*-II in an inverse PCR reaction, a 7.5-kb PCR product was obtained. The amplified genomic DNA was sequenced on both strands using the oligonucleotides *cdp*-I, *cdp*-II, *cdp*-III, *cdp*-IV, *cdp*-V, and *cdp*-VI (see Table 1).

2.5. Construction of the integration vector *pTRK828*

Plasmid *pTRK828* was created and propagated by the ligation of a blunt-end PCR product amplified from genomic *L. acidophilus* NCFM DNA with primers *cdp*-A and *cdp*-B to *pORI28* linearized with *Eco*RV and subsequent transformation into *E. coli* EC1000 (Table 1).

2.6. Genomic integration

The disruption of the genomic target gene was performed as described by Russell and Klaenhammer (2001). The integration event was confirmed by Southern hybridization, performed using the Roche Molecular Biochemicals DIG non-radioactive nucleic acid labeling and detection system. A 496-bp internal *cdp* PCR product was amplified in the presence of digoxigenin-11-dUTP with primers *cdp*-A and *cdp*-B. Hybridization was performed according to the

manufacturer's specifications at 42 °C in standard hybridization buffer plus 50% formamide, and detection was performed with CSPD chemiluminescent substrate.

2.7. Isolation of non-covalently linked cell surface proteins

Proteins located on the cell wall were extracted by treatment with chaotropic salts according to a modified protocol described by Boot et al. (1993). Extracted proteins were further purified using Centricon 30 spin columns (Amicon, Beverly, MA) and resuspended in 100- μ l deionized water. The surface proteins were then separated in an 8% SDS-PAGE.

2.8. Stress challenges

Cultures of *L. acidophilus* were plated onto MRS agar plates supplemented with either NaCl (0.1%, 2%, 2.5% and 3% w/v) or Oxgall (0.5%, 1.0%, 1.5% and 2.0% w/v), and incubated anaerobically for 48 h at 37 °C. Broth cultures propagated to an O.D.₆₀₀ of 0.6 or 1.6, respectively, were also subjected to repeated freezing (−80 °C, 5 min) and thawing (+37 °C, 5 min) cycles and survivors enumerated on MRS agar plates with and without Oxgall (1% w/v). Cells were also exposed to 10% or 15% EtOH for varying times at 37 °C, in MRS broth, and survivors enumerated on MRS agar plates.

2.9. Cell adhesion studies

The adhesion to Caco-2 (ATCC HTB-37) cells was examined as described previously (Chauviere et al., 1992). Briefly, 11-day Caco-2 monolayers were treated with a bacterial suspension at a concentration of $\sim 1 \times 10^8$ bacteria ml⁻¹. Bacteria were incubated on the monolayer for 1.5 h at 37 °C in a mixture of MRS and cell line culture medium (Gibco/Invitrogen, Carlsbad, CA) (ratio 1:2). Following incubation, the monolayers were washed five times with phosphate-buffered saline (PBS), fixed in methanol, Gram-stained, and enumerated with a Nikon Eclipse E600 microscope. Assays were performed in triplicate and adhesion was expressed as percent (%) of wild type.

2.10. Nucleotide sequence accession numbers

The nucleotide sequence presented in this manuscript has been submitted to GenBank and assigned the accession number AY487804.

3. Results

3.1. Identification of a partial open reading frame of *L. acidophilus* NCFM with similarities to *S*-layer genes

The annotated *L. acidophilus* NCFM draft genome sequence revealed a partial ORF, designated ORF_223,

that showed several weak similarities to S-layer proteins of other bacterial species (data not shown). S-layer proteins share a significant degree of similarity and display a number of highly conserved regions. In contrast, the partial *ORF_223* appeared to share only a very limited number of conserved amino acids with S-layer proteins. This indicated that *ORF_223* represented either a new class of S-layer proteins or its gene product (gp) could be located on the outer cell wall. Because both models suggested a possible involvement of *ORF_223* in protection or cell-adhesion, *ORF_223* was targeted for a functional analysis.

3.2. Complete sequence and bioinformatic analysis of the *ORF_223* gene

Double-stranded sequencing of the genomic region including *ORF_223* revealed a gene of 1797 bp, encoding a protein of 599 amino acids with a calculated molecular weight of 64.8 kDa. A potential ρ -independent terminator-like structure with a calculated free energy of -16.84 kcal/mol was identified 10 nucleotides downstream of *ORF_223*. Although a putative -10 -region (5' -TATATT-3') was present 5 nt upstream of the start-codon ATG, no potential -35 -region or ribosomal binding site (RBS) was found. No alternative start-codons with the required transcription signals were observed either in the intergenic region between *ORF_223* and its upstream neighbor *ORF_222* (15 nt), or downstream of the determined start-codon of *ORF_223*. Upstream of *ORF_222*, a potential RBS and poorly conserved -10 - and -35 -regions were identified. However, no DNA secondary structure, potentially with the ability to act as a terminator, was found (data not shown). It appeared that *ORF_222* and *ORF_223* might be co-expressed as part of a larger gene cluster. The intergenic region between *ORF_221* and *ORF_222* amounted to 110 nt and both *ORF_221* and *ORF_220* were oriented in the same direction as *ORF_222*. Preliminary microarray data showed that ORFs 220 through 223 are expressed at similar levels under a variety of conditions, providing some supporting evidence of co-transcription (unpublished data, not shown). A BlastP analysis of *ORF_223* revealed weak similarities to a *Lactobacillus crispatus* S-layer protein (GenBank accession: AAB58734), a *Lactobacillus helveticus* extracellular proteinase (GenBank accession: JC7306), a *Streptococcus thermophilus* cell envelope proteinase (GenBank accession: AAG09771), and a *Streptococcus salivarius* glucosyltransferase (GenBank accession: S22737). Similarities to these protein families were relatively weak ($e > 4 \times 10^{-6}$) and no continuous conserved area larger than 14 amino acids was detected. No Blast similarities were found for the deduced gene-product of the upstream *ORF_222*. Analysis of the global and local Pfam alignments showed two regions in *ORF_223* with similarities to S-layer proteins. Region I from aa 136 to aa

282 and Region II from aa 224 to aa 343 show both hits to the SLAP (pfam03217) domain. Analysis with the BLOCKS and PROSITE databases revealed no conserved regions within the deduced amino acid sequence. Based on these *in silico* analyses, *ORF_223* could not be assigned to a known protein family.

3.3. Phylogenetic classification of *ORF_223*

Based on the Blast and Pfam results, we deduced that *ORF_223* was related to S-layer proteins and potentially involved in cell-wall maturation or degradation. An unrooted phylogenetic tree was generated using protein sequences from related S-layer proteins, cell-wall associated proteinases, peptidoglycan hydrolases, and hexosyltransferases (see Fig. 1). As expected, the three different protein families were grouped into distinct branches of the tree. Despite Blast and Pfam hits for S-layer proteins, *ORF_223* was clearly grouped away from the S-layer protein branch. Instead, *ORF_223* seemed to be a unique entity, positioned in between the S-layer and the peptidoglycan hydrolases, with a slight tendency towards the latter. This suggested an involvement of *ORF_223* in cell-wall processing.

3.4. Inactivation of the *L. acidophilus* *ORF_223* gene

A 500-bp internal region from *ORF_223* was cloned into pORI28 (Law et al., 1995) and electroporated into *L. acidophilus* NCFM carrying pTRK669. One Er^r and Cm^r transformant, carrying both the helper plasmid, pTRK669, and the integration plasmid, pTRK828, was then subjected to a temperature shift at 42°C , to destabilize the helper plasmid. A putative integrant, *L. acidophilus* NCK1375, was recovered and characterized as Cm^r and Er^s , indicating loss of the helper plasmid and integration of pTRK828. Plasmid integration into *ORF_223* was verified by Southern hybridizations using *Bgl*III-restricted DNA (data not shown).

3.5. Localization of the *ORF_223* protein

Proteins non-covalently bound to the outer cell walls of *L. acidophilus* NCFM wild-type and the *ORF_223* knock-out strain, NCK1375, were isolated and subsequently separated using denaturing PAGE (Fig. 2). As expected, both strains clearly showed the dominant S-layer protein fraction with a molecular weight of 44 kDa (Boot et al., 1993). NCK1375, however, lacked one protein band at approximately 63 kDa in comparison to the wild-type strain. The estimated size of this protein band agreed well with the calculated theoretical molecular weight for *ORF_223* at 64.8 kDa.

Proteins located at the cell-surface have to be able to pass through the cell-membrane and, in some cases, through the cell wall. Often, this translocation is mediated

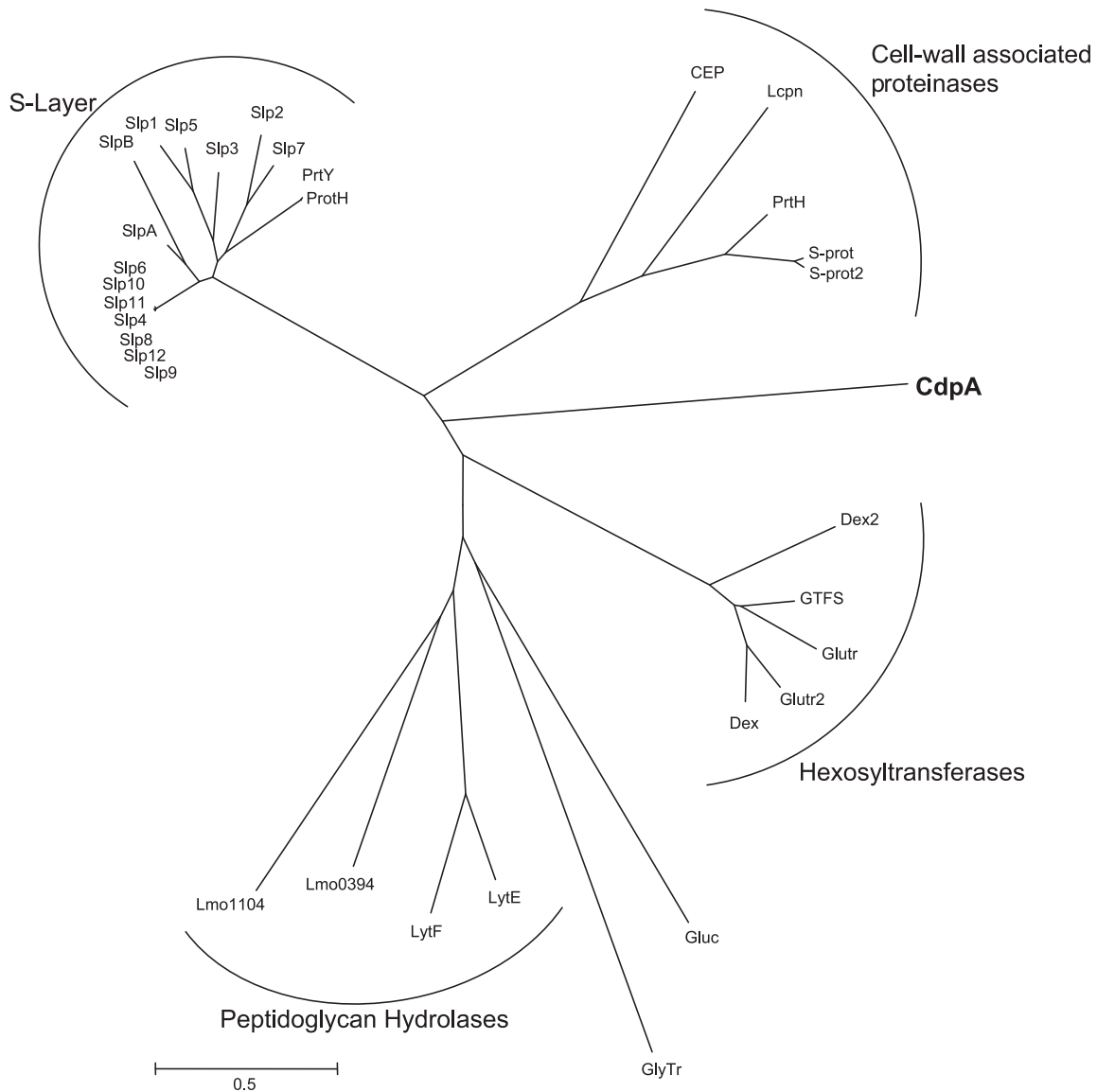


Fig. 1. Unrooted phylogenetic tree of CdpA. Unrooted phylogenetic tree. Based on the PSI-Blast results, protein sequences showing similarities to CdpA were aligned using ClustalX (Blosum30). The unrooted tree was generated using MEGA2.1 software package. Because the identifiers for most S-layer proteins were identical, new designations were chosen to clarify the respective origins. The new protein identifiers and their corresponding references are listed here: CdpA=deduced gene product from *cdpA* (*L. acidophilus*), Slp1=S-layer protein (*Lactobacillus crispatus*), Slp2=silent S-layer protein (*L. crispatus*), Slp3=S-layer protein (*L. crispatus*), Slp4=S-layer protein (*Lactobacillus helveticus*), Slp5=S-layer protein (*L. crispatus*), Slp6=S-layer protein (*L. helveticus*), Slp7=S-layer protein (*L. crispatus*), Slp8=S-layer protein (*L. helveticus*), Slp9=S-layer protein (*L. helveticus*), Slp10=S-layer protein (*L. helveticus*), Slp11=S-layer protein (*L. helveticus*), Slp12=S-layer protein (*L. helveticus*), SlpA=S-layer protein A (*L. acidophilus*), SlpB=S-layer protein B (*L. acidophilus*), Prt-H=proteinase (*L. helveticus*), PrtH=cell envelope-associated proteinase (*L. helveticus*), PrtY=extracellular proteinase (*L. helveticus*), GluTr=Glucosyltransferase (*Streptococcus salivarius*), GluTr2=Glucosyltransferase (*Streptococcus gordonii*), GlyTr=Glycosyltransferase (*Neisseria meningitidis*), GTF-S=Glucosyltransferase (*Streptococcus criceti*), Lcpn=cell surface proteinase (lactocepine) (*Lactobacillus delbrueckii* ssp. *bulgaricus*), Cep=cell envelope proteinase (*Streptococcus thermophilus*), Dex=Dextranase (*Streptococcus mutans*), Dex2=Dextranase (*Leuconostoc mesenteroides*), S-prot=cell-wall associated Serine-proteinase (*Lactococcus lactis* ssp. *cremoris*), S-prot2=cell-wall associated Serine-proteinase (*Lactobacillus paracasei*), Gluc=Endo-1,3-beta-glucanase (*Candida albicans*), LytE=cell-wall hydrolase (*Bacillus subtilis*), LytF=Peptidoglycan hydrolase (*B. subtilis*), Lmo0394=similar to extracellular P60 protein (*Listeria monocytogenes* EGD-e), Lmo1104=similar to extracellular P60 protein (*L. monocytogenes* EGD-e).

by N-terminal signal peptides. *In silico* analyses with the “DAS” transmembrane server (<http://www.sbc.su.se/~miklos/DAS/maindas.html>) showed the potential presence of an N-terminal transmembrane domain. Further analyses using the SignalP Server (Dyrlov et al., 2004) revealed the presence of an N-terminally located signal peptide. Both,

the neural network and the hidden Markov models, trained for gram positive bacteria, predicted a cleavage site at amino acid position 68, reducing its calculated weight to 57 kDa (data not shown). Therefore, ORF_223 appeared to be located in the cell wall and was not covalently bound.

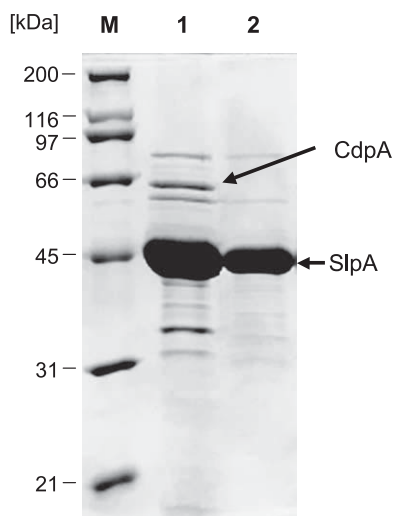


Fig. 2. Localization of the CdpA protein. SDS-PAGE (12%) of the purified cell surface proteins from *L. acidophilus* NCFM and NCK1375, stained with Coomassie-Blue. Lane M: molecular weight standard (Bio-Rad, SDS-PAGE Molecular Weight Standards, Broad Range), lane 1: *L. acidophilus* NCFM wild-type, lane 2: *L. acidophilus* NCK1375. Numbers on the left indicate the molecular masses.

3.6. Growth rate and morphological changes

The growth rates of both the wild-type and the knockout strain were identical during log phase with a generation time of approximately $1.52 \text{ h} \pm 0.03$. Optical densities were measured every 60 min during lag and stationary phases and every 30 min during logarithmic growth (data not shown). *L. acidophilus* NCFM grew as uniform rods and exhibited very little tendency to generate longer cell chains of more than three cells (Fig. 3). These short chains broke up readily upon shearing by vortexing. *L. acidophilus* NCK1375 exhibited the same morphology for single cells, but they existed in extended filamentous chains. Long chains were observed with nearly indistinguishable cells forming stable networks. Both the cell chains and interchain networks were highly resistant to mechanical shearing and were observed throughout all growth phases (Fig. 3). Microarray data indicated that *cdpA* is among the

most highly expressed genes in *L. acidophilus* NCFM and is expressed under a variety of growth conditions and in both early ($\text{O.D.}_{600} \sim 0.3$) and mid log ($\text{O.D.}_{600} \sim 0.6$) phase cells (unpublished data). On a macroscopic level, the sedimentation rate and degree of NCK1375 in broth cultures was significantly higher than NCFM. The sedimented cells appeared to be two to three times more voluminous than the wild-type cell pellets (data not shown). Based on these observations, it appeared that *ORF_223* was involved in cell dechaining and designated as a cell separating protein, *cdpA*.

3.7. Responses to environmental stresses

Wild type *L. acidophilus* NCFM and the *cdpA*⁻ derivative NCK1375 were subjected to a variety of environmental challenges (Fig. 4).

L. acidophilus NCFM was able to tolerate NaCl-concentrations up to 2.5% without any significant loss of viability. At 3.0% NaCl, no growth of *L. acidophilus* NCFM was observed (data not shown). In contrast, at 2% NaCl only 0.02% of the NCK1375 population was recovered.

Challenged with increasing concentrations of Oxgall, *L. acidophilus* NCFM population survived well up to 1.0% Oxgall, then exhibited population losses at 1.5% and 2.0% Oxgall. At concentrations above 1.0%, the colony-morphology of *L. acidophilus* NCFM changed from relatively large, round and white colonies to pinpoint, glassy and crooked. The *cdpA*-knockout strain NCK1375 exhibited more tolerance to 1.5% and 2.0% Oxgall. Interestingly, NCK1375 showed no significant change in colony morphology and, when compared to the wild-type strain, required 24 h less to form detectable colonies on media with Oxgall concentrations greater than 1%.

No significant differences were observed between *L. acidophilus* NCFM and NCK1375 during exposure to 10% EtOH. Extended exposure showed that NCK1375 was more sensitive to 15% EtOH. After 2 h of EtOH exposure, survivors were recovered at 10% of the initial population. After 3-hour exposure to 15% EtOH, the number of survivors was reduced to 0.016%. In contrast, NCFM wild

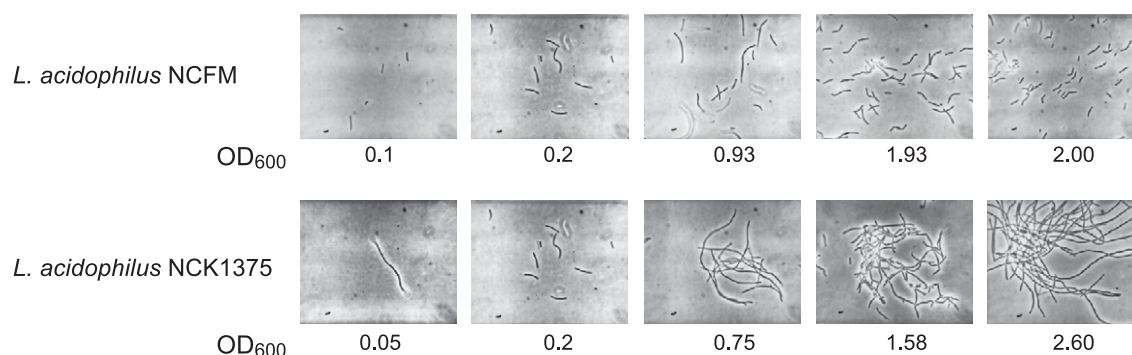


Fig. 3. Morphological appearance throughout the growth cycle. Morphotypes of *L. acidophilus* NCFM (upper row) and NCK1375 (lower row) were sampled at different growth stages and photographed using a Zeiss microscope (Standard 16) with a 500 \times magnification.

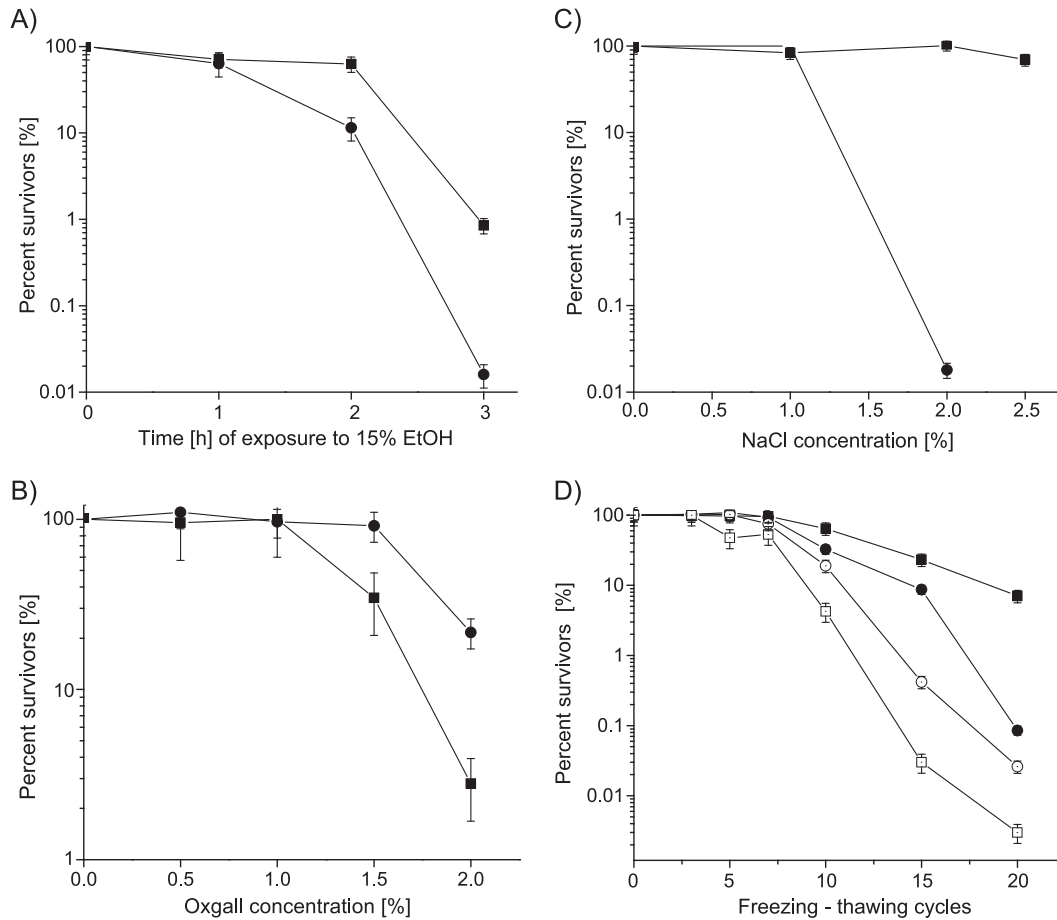


Fig. 4. Stress challenges. Recovery of *L. acidophilus* NCFM and NCK1375 after challenge with varying stresses. Percent survival compared to the rate without stress. (A) Recovery after exposure to 15% EtOH in MRS medium, plated on EtOH-free MRS agar plates. (B) Recovery on NaCl-supplemented MRS agar plates. (C) Recovery on Oxgall-supplemented MRS agar plates. (D) Recovery after repeated freezing-and-thawing cycles. Recovered cells were enumerated on both MRS agar and MRS agar plus 1% (w/v) Oxgall plates. Symbols: *L. acidophilus* NCFM; ● NCK1375; *L. acidophilus* NCFM enumerated on MRS plus 1% Oxgall; ○ NCK1375 enumerated on MRS plus 1% Oxgall.

type was less sensitive to EtOH exposure and survivors were recovered at levels of 63% and 1% after 2 and 3 h, respectively.

Lastly, survival of NCK1375 and *L. acidophilus* NCFM was compared after repeated cycles of freezing (-80°C , 15 min) and thawing (37°C , 5 min) (Fig. 4D). The *cdpA*-knockout strain was more sensitive to freezing–thawing than *L. acidophilus* NCFM. After 20 cycles, 99.2% of the NCK1375 population failed to recover, compared to the levels recovered from *L. acidophilus* NCFM. In contrast, survival of NCK1375 reversed when 1% Oxgall was added to the enumeration medium. In this condition, the more bile-tolerant CdpA-deficient NCK1375 was recovered at higher levels than *L. acidophilus* NCFM over the course of freezing–thawing cycles.

3.8. Cell-adhesion assay

Adhesion of *L. acidophilus* NCFM and NCK1375 to Caco-2 cells was compared. The degree of adhesion for the wild-type strain was defined as 100%. Inactivation of the

cdpA gene and elimination of the CdpA protein resulted in a significant drop in adherence. On average, the knockout strain NCK1375 exhibited only 17.19% ($\pm 7\%$) of the adhesion rate observed for *L. acidophilus* NCFM. For this assay, single cells were enumerated, as the wild-type strain grows either in single cells or in very short chains of two or three cells per chain. The CdpA-deficient strain however, exhibits significant chain growth with an average of at least five to six cells per chain. To eliminate any bias due to the different chain lengths, adhesion levels were recalculated by counting chains as single entities, regardless of their individual cell composition. The adjusted adhesion level for the *cdpA* null strain NCK1375 still remained at only 5.7% of the adhesion of the wild-type strain.

4. Discussion

The *L. acidophilus* NCFM ORF_223, *cdpA*, encodes a protein likely to be involved in the cell division process. Initial PSI-Blast analyses of the deduced amino acid

sequence showed weak similarities to S-layer proteins. In order to test the *in silico* analyses *in vivo*, we inactivated the *cdpA* gene via an integration event. Due to the predicted potential terminator and the presence of highly conserved transcription signals upstream of the adjacent ORF 224 (data not shown), polar effects downstream of the integration event are considered to be unlikely. The most noticeable change of *cdpA* inactivation was a drastic change in morphotype. The *L. acidophilus* wild-type grew as single rod-shaped cells or in very short chains (two to three bacteria), whereas the knockout strain NCK1375 exhibited extended, filamentous, chain growth. Similar morphological effects were found in *Bacillus subtilis* when a peptidoglycan hydrolase, *lytF*, was inactivated (Ohnishi et al., 1999). *LytF* is one of four autolysins (*LytC*, *D*, *E*, and *F*) found in *B. subtilis*. The two minor autolysins *LytE* and *LytF* are expressed during vegetative growth and are found to be involved in the cell separation process. Null-mutants of *lytF* were three to four times longer than the wild-type cells and *lytF cw/F* double mutants exhibited a dense fiber formation, similar to that observed herein for NCK1375. However, a gapped BlastP comparison between *LytF* and *CdpA* revealed no significant sequence similarities. In addition, no conserved domains were shared between the two proteins. Further BlastP analyses revealed weak similarities to cell-wall associated proteinases and hexosyltransferases. Likewise, no conserved domains or other similar regions could be found to either of the two classes. A conserved domain could only be found to S-layer proteins (pfam03217). Besides the S-layer affiliation, a second minor extracellular protease function (O62439, <http://systers.molgen.mpg.de>) was assigned to this domain. The cluster information revealed a PIR classification including similarities to lactocepin, a cell-envelope-associated proteinase commonly found in lactobacilli and lactococci strains.

In silico analyses and predictions of potential transmembrane domains, signal peptides, and signal peptide cleavage sites at the N-terminus of *CdpA* strengthened the prediction this protein might be located on the cell wall. SDS-PAGE analysis of non-covalently bound proteins of the cell wall confirmed the location of *CdpA*.

Despite its BlastP similarity, it is unlikely that *cdpA* is functionally related to S-layer proteins. S-layers are paracrystalline monolayers, covering the whole cell-surface (Boot et al., 1993). For the majority of prokaryotes, the layers are built upon only one protein, although often more than one S-layer gene can be found in an organism (Boot et al., 1995, 1996; Fouet and Mesnage, 2002; Jakava-Viljanen et al., 2002). This requires large amounts of these proteins to be present in order to continuously form and maintain these layers. The amount of protein found for *CdpA* is significantly smaller than the amount of S-layer proteins found on the outer cell-surface. Therefore, it appears unlikely for *CdpA* to be involved in S-layer formation. The change in morphotype and its weak similarities to cell-wall associated proteinases suggest that *CdpA* represents a new enzyme

family involved in the cell separation process, likely by cleaving specific components of the cell wall. The uniqueness of *CdpA* is also reflected by the unrooted phylogenetic tree. *CdpA* was grouped as an entity that was clearly separated from S-layer proteins, autolysins, proteinases, and hexosyltransferases. In addition, no orthologous proteins could be found in the currently available genomic sequences of closely related lactic acid bacteria including *Lactobacillus gasseri* and *Lactobacillus johnsonii* (Pridmore et al., 2004).

To further investigate the effects of the *cdpA* gene inactivation, we applied a series of environmental stresses, and combinations thereof, to both the wild-type and the knockout strain. Bacteria, used as probiotic compounds, in transit through the gastrointestinal tract have to survive the bile secretion of the pancreas and maintain their viability. Unexpectedly, altering cell-wall properties at NCK1375 greatly enhanced its tolerance toward bile salts in comparison to *L. acidophilus* NCFM. One could speculate that these effects might result from a presumed immature structure of the cell wall, where certain components remained crosslinked or uncleaved. This, in turn, may alter the accessibility, uptake, and/or interaction to bile salts, perhaps improving the resistance of the bacteria. The *cdpA*⁻ derivative was also more sensitive to freezing and thawing cycles, perhaps reflecting an immature cell wall due to the inactivation of the *cdpA* gene. Inactivation of the *cdpA* gene dramatically decreased tolerance to NaCl and osmotic stress. These responses to freezing and salt could both reflect the cells' loss of cell-wall integrity and strength. The observed drop in adhesion of the knockout strain agreed well with the functional model of *CdpA*. Proteins involved in adhesion are often linked to the cell wall where they are bound and can form oligomeres (Chhatwal, 2002; Rojas et al., 2002). *CdpA* may be directly involved, but more likely the lack of *CdpA* activity could also result in a loss of anchoring or translocation sites, or the integrity thereof, for adhesion-related surface proteins that are important for the binding capabilities of the wild-type strain.

The specific mechanisms causing the changes in the morphotype, altered responses to selected stresses, and in the capability to adhere to intestinal cells remain unknown. The suggested model of *CdpA* is a cell-wall modifying enzyme that promotes cell division and separation. Elimination of that activity could result in a series of pleiotropic effects that reflect general changes in the cell-wall architecture and/or composition. As genome sequences become available on a number of related lactic acid bacteria, additional analyses will be performed to elucidate whether *CdpA* forms a new class of cell-wall modifying enzymes unique to lactic acid bacteria or strains therein.

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