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Lactobacillus plantarum Extracellular Chitin-Binding Protein and Its Role in the Interaction between Chitin, Caco-2 Cells, and Mucin[▽]†

Borja Sánchez,[1]* Carmen González-Tejedo,[1] Patricia Ruas-Madiedo,[1] María C. Urdaci,[2] and Abelardo Margolles[1]

Instituto de Productos Lácteos de Asturias, Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Ctra. Infiesto s/n, 33300 Villaviciosa, Asturias, Spain,[1] and UMR 5248 CBMN, CNRS-Université Bordeaux 1-ENITAB, Laboratoire de Microbiologie et Biochimie Appliquée, 1 Cours du Général de Gaulle, 33175 Gradignan Cedex, France[2]

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In the present work, we describe the adhesion capabilities of a recombinant *Lactococcus lactis* strain producing an extracellular protein from *Lactobacillus plantarum*. Our results show that this protein may offer the bacterium a mechanism to bind to *N*-acetylglucosamine-containing polymers, such as human mucins, present in different environments.

The lactic acid bacteria (LAB) include thousands of strains that have been part of human nutrition since ancestral times. These microorganisms ferment different kinds of foods, providing the human population with a panoply of fermented products (16). In addition, some LAB strains are considered probiotics, due to their beneficial effects on human health. In this sense, production of extracellular proteins by LAB is a subject of current interest; however, we lack knowledge about the molecular mechanisms through which these microorganisms act on the host. This is partly due to the fact that extracellular proteins are able to directly interact with the mucosal cells, potentially being involved in adhesion to the gut mucosa, host immunomodulation, and molecular cross-talking (9, 11, 14). In particular, interaction with glycoproteins is recognized as a feature explaining the adhesion capabilities of probiotic, commensal, and pathogenic bacteria (10).

In a previous work, we showed that three *Lactobacillus plantarum* strains, including the probiotic strain 299v, secreted several extracellular proteins with an unknown function into the growth culture, and some of these proteins were also expressed in the intestine (12). In addition, other researchers have recently expended considerable effort to identify surface-associated proteins with a potential role in the interaction of *Lb. plantarum* with its surrounding environment (1, 4). Among these proteins, two bind to mucin *in vitro*, and one of these proteins carries a chitin-binding domain (CBP) within its amino acidic sequence. To better characterize how this protein might contribute to the adhesion process, we first cloned the gene encoding the extracellular protein. The *cbp* gene (without the sequence coding for the signal peptide) was ligated to pNZ8110 from *Lactococcus lactis*, a plasmid harboring a promoter inducible by nisin and that allows secretion of the synthesized protein (13). This process yielded the recombinant *Lactococcus lactis* CBP-8 HT strain, which produced a surface-associated CBP (Table 1). All the experimental methods are detailed in file S1 in the supplemental material.

TABLE 1. Strains and primers used in this study

Strain, vector, or primer	Feature(s) or sequence[a]	Reference
Lb. plantarum BMCM12	Natural CBP producer	14
Lc. lactis subsp. *cremoris* NZ9000	Strain MG1363 with *nisRK* genes integrated into the chromosome; host for plasmid pNZ8110 derivatives	11
Lc. lactis subsp. *cremoris* CBP-8 HT	Strain harboring CBP gene from *Lb. plantarum* BMCM12 without the sequence coding for the signal peptide; cloned into pNZ8110; chloramphenicol resistant	This study
pNZ8110	Vector for protein secretion using the signal sequence of the Usp45 protein of *Lc. lactis*; chloramphenicol resistant	11
CBPF	5′-GGGGCGCCGGCCATGGCTTTGTGACGAACCC-3′	This study
CBPHTR	5′-CGTTCGCCGGCCTAGTGATGGTGATGGTGATGCTGTACGTCAATATCTGA-3′	This study

[a] NaeI recognition sites are shown underlined, and the histidine tag is double underlined.

* Corresponding author. Mailing address: Instituto de Productos Lácteos de Asturias, Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Ctra. Infiesto s/n, 33300 Villaviciosa, Asturias, Spain. Phone: 34 985 89 21 31. Fax: 34 985 89 22 33. E-mail: address: borja@ipla.csic.es.

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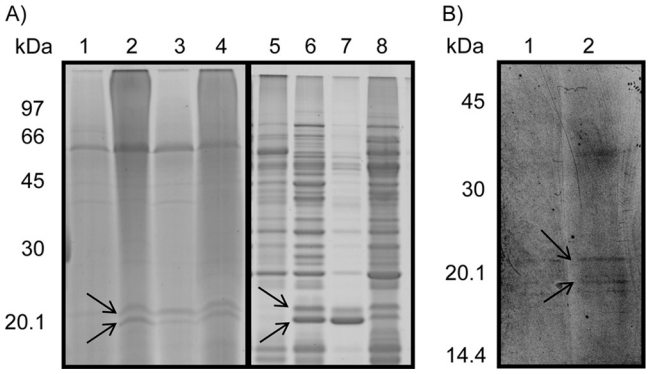

FIG. 1. (A) Representative polyacrylamide gels showing the presence of CBP (arrows) on the supernatants (lanes 1 to 4) and cell wall extracts (lanes 5 to 8) of *Lc. lactis* subsp. *cremoris* CBP-8 HT cultures and cells, respectively. Gene expression was induced by the addition of 40 ng/ml nisin in cultures at an A_{600} of 0.3, and the presence of CBP was investigated at time zero (lanes 1 and 5) and at 3 h (lanes 2 and 6), 6 h (lanes 3 and 7), and 16 h (lanes 4 and 8). (B) Western blot showing the purified His-tagged CBP protein. Ten and 100 ng (lanes 1 and 2, respectively) of the purified protein were bound to polyvinylidene difluoride (PVDF) membranes by electroelution. CBP was visualized using a specific horseradish peroxidase (HRP)-conjugated His tag antibody as described in the text.

Recently, the chitinolytic system of *Lc. lactis* has been described. It consists of a chitinase (β-1,4-poly-*N*-acetyl glucosaminidase; EC 3.2.1.14) and a CBP that resembles the one from *Lb. plantarum* (8). In our experimental design, all the *Lactococcus* strains were grown in GM17 medium, prepared without yeast extract (rich in chitin) to avoid production of its own CBP. Under these conditions, only Usp45 protein, the major protein secreted by *Lc. lactis*, was detected in the spent supernatant (15). In addition, nisin-induced cultures of strain CBP-8 HT did not show measurable β-*N*-acetylglucosaminidase activity (data not shown), which supports the fact that the *Lactococcus* strain used in this work did not induce the chitin utilization system under our experimental conditions. These results are also in agreement with the observation that CBP is not produced by *Lb. plantarum* when yeast extract is removed from the formulation of the culture broth (see file S2 in the supplemental material).

CBP from *Lb. plantarum* is an adhesin when it is displayed on the surface of *Lc. lactis*. CBPs bind to polymeric *N*-acetylglucosamine, attaching the bacteria to the substrate and favoring the degradative actions of chitinases. Due to their bioactive actions, these proteins can interact directly with polymers of *N*-acetylglucosamine, such as the chitin of the cell walls of fungi or the exoskeletons of arthropods. In addition, CBPs favor bacterial adhesion to other *N*-acetylglucosamine-containing polymers, such as those present on intestinal-mucosa surfaces (5, 6, 17).

The recombinant strain *Lc. lactis* CBP-8 HT produced, eminently, a surface-associated CBP (Fig. 1A, lanes 5 to 8), although small amounts of the protein were found in the supernatants as well (Fig. 1A, lanes 1 to 4). This strain showed the best ability to adhere to Caco-2 cell monolayers and mucin,

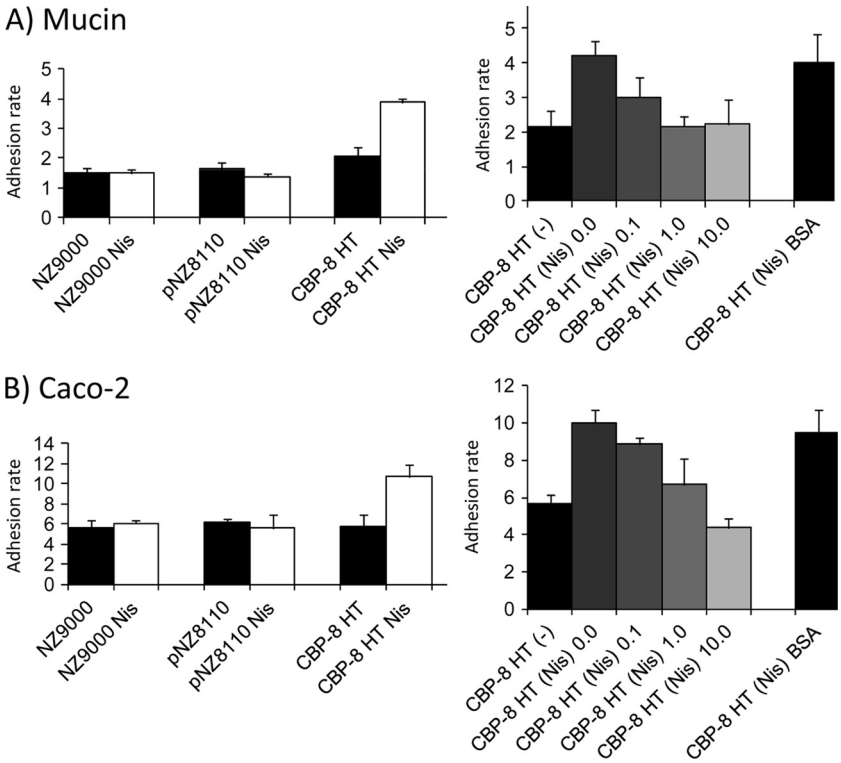

FIG. 2. On the left, adherence of the strains NZ9000, NZ9000-pNZ8110, and CBP-8 HT to mucin (A) and Caco-2 cell monolayers (B), induced (Nis) or not induced with 40 ng/ml nisin. On the right, inhibition of the adhesion of strain CBP-8 HT to mucin and Caco-2 layers with increasing amounts of purified His-tagged CBP protein is shown (0, 0.1, 1.0 and 10.0 μg). Ten micrograms of bovine serum albumin (BSA) was used as a negative control. Results are expressed as the means ± standard deviations of the results from three independent biological triplicates.

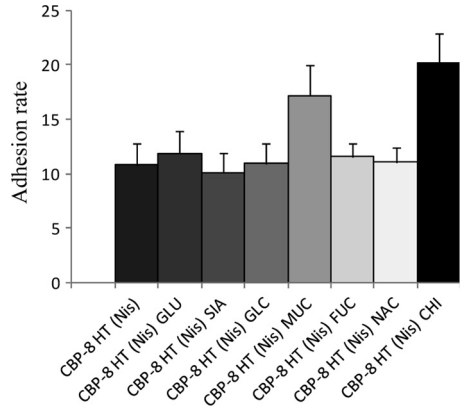

FIG. 3. Rates of adhesion to Caco-2 cell layers of strain CBP-8 HT induced with 40 ng/ml nisin in the presence of different sugars; the sugars had been preincubated with the monolayer cells for 30 min. GLU, glucose; SIA, sialic acid; GLC, glucuronic acid; MUC, mucin; FUC, fucose; NAC, *N*-acetylglucosamine; CHI, chitobiose. Results are expressed as the means ± standard deviations of results from three independent biological triplicates.

the process being inhibited in a dose-dependent manner if the purified CBP protein was included in the experiment (Fig. 2). This suggested that CBP could block some common receptor molecules on the surfaces of mucin and Caco-2 cells. In this way, CBPs are nonhydrolytic accessory proteins essential for chitin degradation that bind to *N*-acetylglucosamine present in a wide variety of polymers, including chitin and mucins (5–7).

***Lc. lactis* CBP-8 HT binds to chitin, as well as to chitobiose or mucin immobilized on the surfaces of Caco-2 cells.** Adhesion assays in which mucin or Caco-2 cell monolayers were preincubated with different sugars, all of them components of the polymers present in the gastrointestinal tract mucosa, were performed (see file S1 in the supplemental material). No variations in the adhesion ability of the strain were observed with mucin as a matrix (data not shown). In contrast, significant increases in its adhesion appeared when the Caco-2 cell monolayers were preincubated with mucin or chitobiose (Fig. 3). The rest of the sugars, including *N*-acetylglucosamine, failed to influence the adhesion of the recombinant strain to Caco-2 cells, perhaps because they were unable to be retained on the Caco-2 cell monolayers or because they do not interact at all with CBP. Further experiments including immobilized sugars will identify whether CBP is able to interact with other monosaccharides or not. In fact, it has recently been described that the use of an intestinal cell monolayer together with a mucin solution is a convenient approach to mimic the physiological environment occurring in the gastrointestinal tract (10). As in our experiments, the presence of mucin increased, in general, bacterial adhesion to Caco-2 cell monolayers, giving higher values than those obtained with the mucin-producing cell line HT29-MTX (10). We also tried to assay the Caco-2 cells pretreated with chitin, but we did not succeed since the chitin is dissolved in methanol, which severely affects the properties of the monolayers. Thus, to better support our findings, adhesion experiments using only chitin as the coated molecule were conducted. The adhesion of strain CBP-8 HT was significantly greater than that of its respective controls (Fig. 4). These data strongly suggested that CBP could be a protein involved in binding to the *N*-acetylglucosamine residues.

CBP as a specific colonization factor of *Lb. plantarum*. The sequence of *cbp* from *Lb. plantarum* BMCM12, cloned in *Lc. lactis*, was very similar to those from other *Lb. plantarum* strains, *Lactobacillus sakei* subsp. *sakei* 23K, *Enterococcus* sp., *Bacillus* sp., and *Listeria* sp. (see file S3 in the supplemental material). Remarkably, no homologous sequences were found in other *Lactobacillus* species (with the above-mentioned exception), suggesting that CBP might have been acquired by the *Lb. plantarum* species in a horizontal gene transfer event.

Homologous proteins harboring this domain are found in other bacteria, and they are frequently proposed as important colonization factors. Probably the best known example is given by the CBP from *Vibrio cholerae*, the causative agent of cholera. In this microorganism, this protein has been described as a colonization factor involved in the adhesion to the chitinous exoskeleton of *Daphnia magna*, a member of the zooplankton community present in regions of cholera endemicity, and to Caco-2 cells (7). CBP thus provides the pathogen with a molecular mechanism that links its natural habitat with the intestinal surface of the human host.

To sum up, our results provide substantial evidence that CBP is able to directly bind to *N*-acetylglucosamine residues contained in chitin and in glycoproteins present on the surfaces

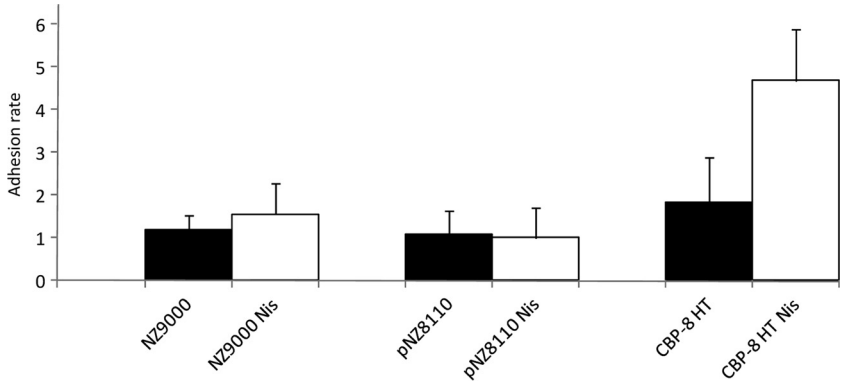

FIG. 4. Rates of adhesion of strains NZ9000, NZ9000-pNZ8110, and CBP-8 HT, induced (Nis) or not induced with 40 ng/ml nisin, to chitin obtained from shrimp shells previously coated on the wells of a Maxisorp plate. Results are expressed as the means ± standard deviations of results from three independent biological triplicates.

of intestinal mucins and epithelial cells (2, 3). This protein could thus perform important roles in the extracellular biology of *Lb. plantarum*, allowing adhesion to different surfaces present in different environments. Further research using immobilized glycosidic compounds, such as Lewis antigens or other physiologically relevant oligosaccharides, will increase our knowledge on the precise interaction of CBP with the glycoproteins present in the human gut.

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