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Functional Analysis of the p40 and p75 Proteins from *Lactobacillus casei* BL23

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Key Words

Lactobacillus · Probiotic · Cell-wall hydrolases

Abstract

The genomes of *Lactobacillus casei/paracasei* and *Lactobacillus rhamnosus* strains carry two genes encoding homologues of p40 and p75 from *L. rhamnosus* GG, two secreted proteins which display anti-apoptotic and cell protective effects on human intestinal epithelial cells. p40 and p75 carry cysteine, histidine-dependent aminohydrolase/peptidase (CHAP) and NLPC/P60 domains, respectively, which are characteristic of proteins with cell-wall hydrolase activity. In *L. casei* BL23 both proteins were secreted to the growth medium and were also located at the bacterial cell surface. The genes coding for both proteins were inactivated in this strain. Inactivation of LCABL_00230 (encoding p40) did not result in a significant difference in phenotype, whereas a mutation in LCABL_02770 (encoding p75) produced cells that formed very long chains. Purified glutathione-S-transferase (GST)-p40 and -p75 fusion proteins were able to hydrolyze the muropeptides from *L. casei* cell walls. Both fusions bound to mucin, collagen and to intestinal epithelial cells and, similar to *L. rhamnosus* GG p40, stimulated epidermal growth factor receptor phosphorylation in mouse intestine ex vivo. These results indicate that extracellular proteins belonging to the machinery of cell-wall metabolism in the closely related *L. casei/paracasei-L. rhamnosus* group are most likely involved in the probiotic effects described for these bacteria

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Introduction

Lactobacilli are normal constituents of the intestinal tract microbiota and some strains have attracted intense interest due to their probiotic health-promoting effects. The adaptation to the intestinal tract of these bacteria is based on two different aspects. First, they have developed strategies to adapt to the harsh conditions found in this niche, including mechanisms to cope with stress (i.e. low pH, presence of bile), adhere to the mucosal surface (i.e. epithelial cells, mucus and extracellular matrix protein binding) and exploit the nutritional characteristics of the medium (e.g. utilization of different carbohydrates) [Lebeer et al., 2008]. Second, regular presence in the human gut provided these bacteria with multiple mechanisms to interact with the host that are at the basis of their health-promoting effects. Intestinal epithelial and immune cells are responsive to probiotic-derived products (metabo-

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lites, proteins, cell-wall constituents or DNA) that are important for the maintenance of intestinal homeostasis, acting at the level of epithelial barrier protection and immunomodulation [Corthesy et al., 2007; Lebeer et al., 2008; Vanderpool et al., 2008; Hörmannsperger and Haller, 2010]. Most of the characterized probiotic-host interactions take place through pattern recognition receptors (e.g. toll-like receptors, TLR) which recognize common molecules present at the bacterial surface (peptidoglycan, lipoteichoic acids) or derived from them (e.g. unmethylated CpG DNA, exopolysaccharides) [Lebeer et al., 2010]. In addition, other secreted or cell surface-exposed probiotic factors have been described, but their nature and targets are poorly known. The GroEL chaperone from *Lactobacillus johnsonii* La1 is surface located and stimulates interleukin-8 secretion in macrophages [Bergonzelli et al., 2006]. The surface-layer protein A from *Lactobacillus acidophilus* NCFM binds to the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor in dendritic cells influencing interleukin production [Konstantinov et al., 2008]. Soluble low-molecular-weight peptides produced by *Lactobacillus rhamnosus* GG (LGG), one of the most intensively studied probiotic strains, activate MAP kinases and induce cytoprotective heat-shock proteins in intestinal cells [Tao et al., 2006]. Yan and Polk [2002] reported that LGG prevented cytokine-induced apoptosis in intestinal epithelial cells. In fact, the factors responsible for this were present in the LGG culture supernatant and have been identified as two proteins with approximate sizes of 40 and 75 kDa (p40 and p75, respectively). Purified p40 and p75 stimulate Akt activation, display anti-apoptotic activity, prevent epithelial barrier damage caused by oxidative agents and decrease susceptibility to dextran sodium sulfate-induced colon epithelial injury in mice [Yan et al., 2007, 2010; Seth et al., 2008]. The complete and the partial ORFs encoding p40 and p75 from LGG were determined and showed to code, respectively, for a protein of unknown function and a protein annotated as a putative cell-wall hydrolase [Yan et al., 2007]. In this work, we show that genes encoding homologues to these proteins are also present in other strains of the closely related *Lactobacillus casei/paracasei-L. rhamnosus* group. We have characterized p40 and p75 proteins from *Lactobacillus casei* BL23, a strain widely used in genetic and physiologic studies and which displays probiotic effects in animal models [Foligne et al., 2007; Monedero et al., 2007; Rochat et al., 2007]. We have shown that these proteins play a cell-wall hydrolytic role in this host.

Results

p40- and p75-Encoding Genes Are Present in the Genomes of Several Lactobacillus Strains

Yan et al. [2007] reported that Western blot analysis with anti-p40 and anti-p75 sera evidenced the presence of p40 and p75 homologue proteins in *L. casei* ATCC393 and *L. casei* ATCC334 but not in *L. acidophilus*. In fact, a genomic BLAST search of sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) revealed that genes encoding p40 and p75 homologues were exclusively found in the genomes of *L. casei/paracasei* and *L. rhamnosus* strains. Sequence alignment of p40 (fig. 1a) shows that, with the exception of a serine-rich region (amino acids 256–286 in p40 from *L. casei* BL23), this protein exhibits more than 98% identity within *L. casei/paracasei* and *L. rhamnosus* strains. Analysis of p40 domain composition revealed a C-terminal CHAP domain (cysteine, histidine-dependent aminohydrolase/peptidase, PF05257) which is usually present in N-acetylmuranoyl-L-alanine amidases or endopeptidases involved in cell-wall metabolism [Bateman and Rawlings, 2003]. The p40 N-terminal portion comprised a domain of unknown function (COG3883) with low similarity (about 26–30% amino acid identity) to proteins showing cell-wall binding or peptidoglycan hydrolytic activity from *Bacillus*, *Clostridium*, *Streptococcus* or *Listeria* species.

p75 homologue proteins were encoded by genes whose translation products had a molecular weight of 49 kDa. The reported size for the LGG protein was 75 kDa, for which it was assumed that p75 displayed an anomalous migration in SDS-PAGE gels. Domain analysis revealed that p75 was also a modular protein carrying a C-termi-

Fig. 1. a Amino acid alignment of p40 proteins from *L. casei* BL23 (LCABL_00230), *L. casei* ATCC334 (LSEI_0020), *L. paracasei* ATCC25302 (HMPREF0530_0772), *L. paracasei* 8700:2, *L. rhamnosus* GG (LGG_00031), *L. rhamnosus* Lc705 (LC705_00025), *L. rhamnosus* LMS2-1 (HMPREF0539_0332) and *L. rhamnosus* HN001 (LRH_09303). **b** Amino acid alignment of p75 proteins from *L. casei* BL23 (LCABL_02770), *L. casei* ATCC334 (LSEI_0281), *L. paracasei* ATCC25302 (HMPREF0530_0378), *L. paracasei* 8700:2, *L. rhamnosus* GG (LGG_00324), *L. rhamnosus* Lc705 (LC705_00310), *L. rhamnosus* LMS2-1 (HMPREF-0539_2610) and *L. rhamnosus* HN001 (LRH_12224). Numbers in parentheses refer to the gene locus encoding p40 or p75, except for *L. paracasei* 8700:2 which does not possess an annotated genome (GenBank ABQV00000000). The position of the putative cleavage sites for signal peptidase I are marked with an arrow. The extension of the different protein domains is indicated by horizontal dark lines.

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             Signal peptide  ▼                    COG3883
BL23        MKFNKVMITLVAAVTLAGSASAVTPVFADTSDSIASNKSETNALLKQIEDANTEVINLNKQIDTKNGQISDATAKISATDAKISSLSGEIVAAQKNVTARKNNLKDQLIS   110
ATCC334     MKFNKVMITLVAAVTLAGSASAVTPVFADTSDSIASNKSETNALLKQIEDANTEVINLNKQIDTKNGQISDATAKISATDAKISSLSGEIVAAQKNVTARKNNLKDQLIS   110
ATCC25302   MKFNKVMITLVAAVTLAGSASAVTPVFADTSDSIASNKSETNALLKQIEDANTEVINLNKQIDTKNGQISDATAKISATDAKISSLSGEIVAAQKNVTARKNNLKDQLIS   110
8700:2      MKFNKVMITLVAAVTLAGSASAVTPVFADTSDSIASNKSETNALLKQIEDANTEVINLNKQIDTKNGQISDATAKISATDAKISSLSGEIVAAQKNVTARKNNLKDQLIS   110
LGG         MKFNKAMMTLVAAVTLAGSVSALTPVFADTSASIASNKSETNDLLKQIEAANTEVINLNKQIDAKNGEISDATAKISATDAKIASLSGEITAAQKNVAARKNNLKDQLIS   110
Lc705       MKFNKAMMTLVAAVTLAGSVSAVTPVFADTSASIASNKSETNDLLKQIEAANTEVINLNKQIDAKNGEISDATAKISATDAKIASLSGEITAAQKNVAARKNNLKDQLIS   110
LMS2-1      MKFNKAMMTLVAAVTLAGSVSAVTPVFADTSASIASNKSETNDLLKQIEAANTEVINLNKQIDAKNGEISDATAKISATDAKIASLSGEITAAQKNVAARKNNLKDQLIS   110
HN001       MKFNKAMMTLVAAVTLAGSVSAVTPVFADTSASIASNKSETNDLLKQIEAANTEVINLNKQIDAKNGEISDATAKISATDAKIASLSGEITAAQKNVAARKNNLKDQLIS   110

                                            COG3883
BL23        LQKKAGNSVSGNVYIDFVLNSQNLSDLIARTMTVGKLSQASKDALDAVTVAKDKLAGLKTEQENARQTLVSTKASLETQKSQLVSLQKDANDKQDALNKQIADHKDELVA   220
ATCC334     LQKKAGNSVSGNVYIDFVLNSQNLSDLIARTMTVGKLSQASKDALDAVTVAKDKLAGLKTEQENARQTLVSTKASLETQKSQLVSLQKDANDKQDALNKQIADHKDELVA   220
ATCC25302   LQKKAGNSVSGNVYIDFVLNSQNLSDLIARTMTVGKLSQASKDALDAVTVAKDKLAGLKTEQENARQTLVSTKASLETQKSQLVSLQKDANDKQDALNKQIADHKDELVA   220
8700:2      LQKKAGNSVSGNVYIDFVLNSQNLSDLIARTMTVGKLSQASKDALDAVTVAKDKLAGLKTEQENARQTLVSTKASLETQKSQLVSLQKDANDKQDALNKQIADHKDELVA   220
LGG         LQKKAGSSVSGNVYIDFVLNSQSLSDLIARTMTVGKLSQASKDALDAVTVAKDKLAALKSEQETARQTLVSTKASLETQKSQLETLQKTASDKQDALNKEIADHKDELVA   220
Lc705       LQKKAGSSVSGNVYIDFVLNSQSLSDLIARTMTVGKLSQASKDALDAVTVAKDKLAALKSEQETARQTLVSTKASLETQKSQLETLQKTASDKQDALNKEIADHKDELVA   220
LMS2-1      LQKKAGSSVSGNVYIDFVLNSQSLSDLIARTMTVGKLSQASKDALDAVTVAKDKLAALKSEQETARQTLVSTKASLETQKSQLETLQKTASDKQDALNKEIADHKDELVA   220
HN001       LQKKAGSSVSGNVYIDFVLNSQSLSDLIARTMTVGKLSQASKDALDAVTVAKDKLAALKSEQETARQTLVSTKASLETQKSQLETLQKTASDKQDALNKEIADHKDELVA   220

                                                                                                CHAP
BL23        LQSQFAQEQSEAAAATQAALKTVAASTASSSTTSSS.....SSATSTSTIANNTSSSSNNSSSIINNNTTSGSGSHADYSSSGNTYPWGQCTWYVKSVASWAGNGWGNGA   325
ATCC334     LQSQFAQEQSEAAAATQAALKTVAASTASSSTTSSS.....SSATSTSTIA...............NNTASGSGSHADFSGSGNTYPWGQCTWYVKSVAPWAGNGWGNGA   310
ATCC25302   LQSQFAQEQSEAAAATQAALKTVAASTASSSTTSSS.....SSATSTSTIANNTSSSSNNSSSVITNNTASGSGSHADFSGSGNTYPWGQCTWYVKSVAPWAGNGWGNGA   325
8700:2      LQSQFAQEQSAAAAATQAALKTVAASTASSSTTSSS.....SSATSTSTIANNTSSSSNNSSSVITNNTASGSGSHADFSGSGNTYPWGQCTWYVKSVAPWAGNGWGNGA   325
LGG         LQSQFAQEQSEAAKATQAALKTAAASTASSSTSSTSNKSANSSVLSTGTSSTNTSSNSGASSTVISSNTASGSGSHADYSGSGNTYPWGQCTWYVKSVASWAGNGWGNGA   330
Lc705       LQSQFAQEQSEAAKATQAALKTAAASTASSSTSSTSNKSANSSVLSTGTSSTNTSSNSGASSTVISSNTASGSGSHADYSGSGNTYPWGQCTWYVKSVASWAGNGWGNGA   330
LMS2-1      LQSQFAQEQSEAAKATQAALKTAAASTASSSTSSTSNKSANSSVLSTGTSSTNTSSNSGASSTVISSNTASGSGSHADYSGSGNTYPWGQCTWYVKSVASWAGNGWGNGA   330
HN001       LQSQFAQEQSEAAKATQAALKTAAASTASSSTSSTSNKSANSSVLSTGTSSTNTSSNSGASSTVISSNTASGSGSHADYSGSGNTYPWGQCTWYVKSVASWAGNGWGNGA   330

                                 CHAP
BL23        QWGSSAAAAGFTVNHTPAAGSIIVFAAGQSVGGQWTADGSYGHVAYVQSVSGDSVTISQGGMGFSSPTGPNTQTISGASSYVYIH   410
ATCC334     QWGGSAAAAGFTVNHTPAAGSIIVFAAGQSVGGQWTADGSYGHVAYVQSVSGDSVTISQGGMGFSSPTGPNTQTISGASSYVYIH   395
ATCC25302   QWGGSAAAAGFTVNHTPAAGSIIVFAAGQSVGGQWTADGSYGHVAYVQSVSGDSVTISQGGMGFSSPTGPNTQTISGASSYVYIH   410
8700:2      QWGSSAAAAGFTVNHTPAAGSIIVFAAGQSVGGQWTADGSYGHVAYVQSVSGDSVTISQGGMGFSSPTGPNTQTISGASSYVYIH   410
LGG         EWGASAAAAGFTVNHTPAAGSIIVFAAGQSVGGQWTADGSYGHVAYVQSVSGDSVTITQGGMGFSSPTGPNTQTISGASSYVYIH   415
Lc705       EWGASAAAAGFTVNHTPAAGSIIVFAAGQSVGGQWTADGSYGHVAYVQSVSGDSVTITQGGMGFSSPTGPNTQTISGASSYVYIH   415
LMS2-1      EWGASAAAAGFTVNHTPAAGSIIVFAAGQSVGGQWTADGSYGHVAYVQSVSGDSVTITQGGMGFSSPTGPNTQTISGASSYVYIH   415
HN001       EWGASAAAAGFTVNHTPAAGSIIVFAAGQSVGGQWTADGSYGHVAYVQSVSGDSVTITQGGMGFDSPTGPNTQTISGASSYVYIH   415
a
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             Signal peptide      ▼
BL23        MVDAKKVLSVTAGFVGAAGLATLATGANTVSASTGTVNYKSGATTVWNSPSWHQVKRYVTFGDKVQVLGQKVDRNGATWYKVGDNQWIPLIYLNFDGKTVTVQAPEQTAS   110
ATCC334     MVDAKKVLSVTAGFVGAAGLATLATGANTVSASTGTVNYKSGATTVWNSPSWHQVKRYVTFGDKVQVLGQKVDRNGATWYKVGDNQWIPGIYLNFDGKTVTVQAPEQTAS   110
ATCC25302   MVDAKKVLSVTAGFVGAAGLATLATGANTVSASTGTVNYKSGATTVWNSPSWHQVKRYVTFGDKVQVLGQKVDRNGATWYKVGDNQWIPGIYLNFDGKTVTVQAPEQTAS   110
8700:2      MVDAKKVLSVTAGFVGAAGLATLATGANTVSASTGTVNYKSGATTVWNSPSWHQVKRYVTFGDKVQVLGQKVDRNGATWYKVGDNQWIPGIYLNFDGKTVTVQAPEQTAS   110
LGG         MVDSKKVLSVTAGFVGAAGLAALATGANTVSASTGTVSYKSGATTVWNSPSWHQVKRYVTFGDTVQLLGKTVDQNGATWYKVGDNQWIPELYLNVAGKTATVETPSSAAS   110
Lc705       MVDSKKVLSVTAGFVGAAGLAALATGANTVSASTGTVSYKSGATTVWNSPSWHQVKRYVTFGDTVQLLGKTVDQNGATWYKVGDNQWIPELYLNVAGKTATVETPSSAAS   110
LMS2-1      MVDSKKVLSVTAGFVGAAGLAALATGANTVSASTGTVSYKSGATTVWNSPSWHQVKRYVTFGDTVQLLGKTVDQNGATWYKVGDNQWIPELYLNVAGKTATVETPSSAAS   110
HN001       MVDSKKVLSVTAGFVGAAGLAALATGANTVSASTGTVSYKSGATTVWNSPSWHQVKRYVTFGDTVQLLGKTVDQNGATWYKVGDNQWIPELYLNVAGKTATVETPSSAAS   110

BL23        QAPVSQAPASQAPASQAPASQ..AAAQPDTQTANIQLYVKNIGSAVTVWATPAYSQATGQYLEGNQTLTAVAQLQANGETWYRLANGGYVPERFAST.....TPAPAPQS   213
ATCC334     QAPVSQAPASQAPASQAPASQ..AAAQPDTQTANIQLYVKNIGSAVTVWATPAYSQATGQYLEGNQTLTAVAQLQANGETWYRLANGGYVPERFAST.....TPAPAPQS   213
ATCC25302   QAPVSQAPASQAPASQAPASQ..AATQPDTQTANIQLYVKNIGSAVTVWATPAYSQATGQYLEGNQTLTAVAQLQANGETWYRLANGGYVPERFAST.....TPAPAPQS   213
8700:2      QAPVSQAPASQAPASQAPASQ..AAAQPDTQTANIQLYVKNIGSAVTVWATPAYSQATGQYLEGNQTLTAVAQLQANGETWYRLANGGYVPERFAST.....TPAPAPQS   213
LGG         QTAVSQAPASQAPTSQAPATQTPAAPQTDTQTANTQLYVKNIGSAVTVWTTPAYTHATGQYLEGSQTLTAVAQQQANGETWYRLANGGYVPARFVSTTPVAVTPQPAAPQ   220
Lc705       QTAVSQAPASQAPTSQAPATQTPAAPQTDTQTANTQLYVKNIGSAVTVWTTPAYTHATGQYLEGSQTLTAVAQQQANGETWYRLANGGYVPARFVSTTPVAVTPQPAAPQ   220
LMS2-1      QTAVSQAPASQAPTSQAPATQTPAAPQTDTQTANTQLYVKNIGSAVTVWTTPAYTHATGQYLEGSQTLTAVAQQQANGETWYRLANGGYVPARFVSTTPVAVTPQPAAPQ   220
HN001       QTAVSQAPASQAPTSQAPATQTPAAPQTDTQTANTQLYVKNIGSAVTVWTTPAYTHATGQYLEGSQTLTAVAQQQANGETWYRLANGGYVPARFVSTTPVAVTPQPAAPQ   220

BL23        SAATSVAAPTAPVSDATASNAAASNAAASDVAVSSAAASSAA...........ASLAAASE.....AAAVANASSAAASSAA...AVASAAAESSAAAESSAAASKAAAD   304
ATCC334     SAATSVAAPTAPVSDATASNAAASNAAASDAAVSSAAASSAA...........ASLAAASE.....AAAVANASSAAASSAA...AVASAAAESSAAAESSAAASRAAAD   304
ATCC25302   SAATSVAAPTAPVSDATASNAAASNAAASDAAVSSAAASSAA...........ASSAAASS.....AA....ASSAAASSAAASLAVASEAAASVANA.SSAAASS.AAD   301
8700:2      SAATSVAAPTAPVSDATASNAAASNAAASDVAVSSAAASSAA...........ASLAAASE.....AAAVANASSAAASSAA...AVA......SAAAESSAAASKAAAD   298
LGG         SNEASVASTNTNAANDS...AAASSAAASQAAASSAAASTAAANAAVASANATASQAAASEAAASQAA..ASQAAASQAAASQAAASQAAASQAAASQAAASQAAASQAA   325
Lc705       SNEASVASTNTNAANDS...AAASSAAASQAAASSAAASTAAANAAVASANAIASQAAASE.....AA..ASQAAASQAAASQAAASQAAASQAAASQAAASQAAASQAA   320
LMS2-1      SNEASVASTNTNAANDS...AAASSAAASQAAASSAAASTAAANAAVASANAIASQAAASE.....AA..ASQAAASQAAASQAAASQAAASQAAASQAAASQAAASQAA   320
HN001       SNEASVASTNTNAANDS...AAASSAAASQAAASSAAASTAAANAAVASANATASQAAASEAAASQAA..ASQAAASQAAASQAAASQAAASQAAASQAAASQAAASQAA   325

                                                                                                  NLPC/P60
BL23        SSAAAVQTTTPESSAAPATTQVDATQEQQQQAEPSNTVNTEETTNNATP.......TPAPTPTPAPTPAPAPVTPSRQAKIQAVISIAEQQIGKPYVWGGKGPNSFDCSG   407
ATCC334     SSAAAVQTTTPESSAAPATTQVDATQEQQQQAEPSNTVNAEETTNNA.........TPAPTPTPAPTPAPAPVTPSRQAKIQAVISIAEQQIGKPYVWGGKGPNSFDCSG   405
ATCC25302   SSAAAVQTTTPESSAAPATTQVDATQEQQQQAEPSNTVNAEETTNNA.TPTPAPTPTPAPTPTPAPTPAPAPVTPSRQAKIQAVISIAEQQIGKPYVWGGKGPNSFDCSG   410
8700:2      SSAAAVQTTTPESSAAPATTQVDATQEQQQQAEPSNTVNTEETTNNATP.......TPAPTPTPAPTPAPAPVTPSRQAKVQAVISIAEQQIGKPYVWGGKGPNSFDCSG   401
LGG         ASQAAANAAQQAPANQANV.......................TTTQV.NANQAQQQTATATPAVNTSNQTAAVSASRQAKIQAVIAIAEQQVGKPYVWGGKGPNSFDCSG   411
Lc705       ASQAAANAAQQAPANQANV.......................TTTQV.NANQAQQQTATATPAVNTSNQTAAVSASRQAKIQAVIAIAEQQVGKPYVWGGKGPNSFDCSG   406
LMS2-1      ASQAAANAAQQAPANQANV.......................TTTQV.NANQAQQQTATATPAVNTSNQTAAVSASRQAKIQAVIAIAEQQVGKPYVWGGKGPNSFDCSG   406
HN001       ASQAAANAAQQAPANQANV.......................TTTQV.NANQAQQQTATATPAVNTSNQTAAVSASRQAKIQAVIAIAEQQVGKPYVWGGKGPNSFDCSG   411

                           NLPC/P60
BL23        LMYYAFLNGAGVNIGGWTVPQESSGQQVSLSALQPGDLLFWGGHGSSYHVALYIGGGTMIQAPQPGENVKYTALAYFMPDFAVRPS   493
ATCC334     LMYYAFLNGAGVNIGGWTVPQESSGQQVSLSALQPGDLLFWGGHGSSYHVALYIGGGTMIQAPQPGENVKYTALAYFMPDFAVRPS   491
ATCC25302   LMYYAFLNGAGVNIGGWTVPQESSGQQVSLSALQPGDLLFWGGHGSSYHVALYIGGGTMIQAPQPGENVKYTALAYFMPDFAVRPS   496
8700:2      LMYYAFLNGAGVNIGGWTVPQESSGQQVSLSALQPGDLLFWGGHGSSYHVALYIGGGTMIQAPQPGENVKYTALAYFMPDFAVRPS   487
LGG         LMYYAFLNGAGVNIGGWTVPQESSGTQVSLSALQPGDLLFWGSHGSTYHVALYIGGGTMIQAPQPGENVKYTALAYFMPDFAVRPS   497
Lc705       LMYYAFLNGAGVNIGGWTVPQESSGTQVSLSALQPGDLLFWGSHGSTYHVALYIGGGTMIQAPQPGENVKYTALAYFMPDFAVRPS   492
LMS2-1      LMYYAFLNGAGVNIGGWTVPQESSGTQVSLSALQPGDLLFWGSHGSTYHVALYIGGGTMIQAPQPGENVKYTALAYFMPDFAVRPS   492
HN001       LMYYAFLNGAGVNIGGWTVPQESSGTQVSLSALQPGDLLFWGSHGSTYHVALYIGGGTMIQAPQPGENVKYTALAYFMPDFAVRPS   497
b
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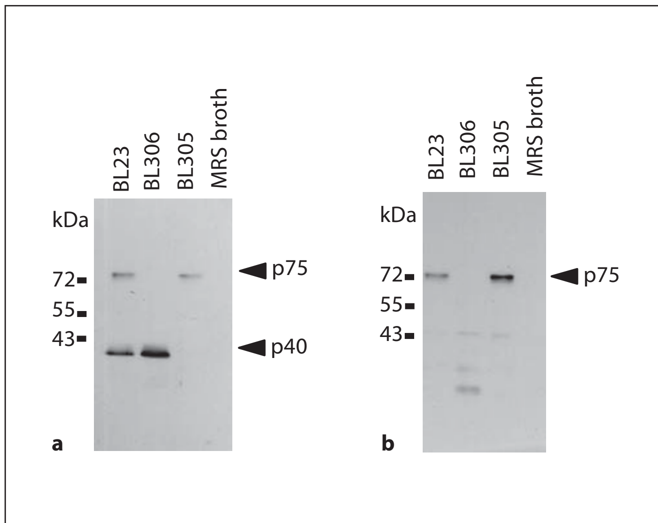

Fig. 2. p40 and p75 are secreted in *L. casei* BL23. **a** Western blot with anti-p40 serum. **b** Western blot with anti-p75 serum. *L. casei* supernatants (10 μl) or fresh MRS broth (10 μl) were loaded in each well. BL23 (wild type); BL305 (LCABL_00230::pRV300); BL306 (LCABL_02770:: pRV300).

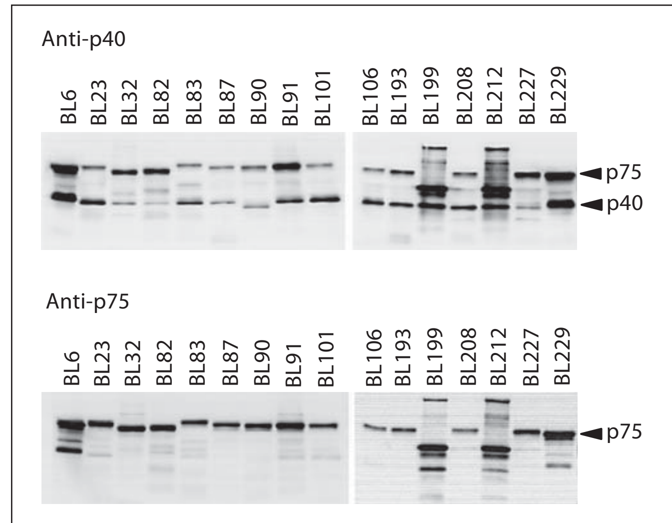

Fig. 3. Western blot screening for the presence of p40 and p75 in the supernatants of several *L. casei/paracasei* strains. 20 μl of bacterial overnight culture supernatants were subjected to SDS-PAGE and probed with antisera raised against LGG p40 and p75.

nal NLPC/P60 (PF00877) domain and an N-terminal domain of unknown function (fig. 1b). The NLPC/P60 domain is usually found at the C-terminus of bacterial proteins described as putative cell-wall hydrolases of the endopeptidase class and in invasion-associated proteins from pathogens [Anantharaman and Aravind, 2003]. p75 from *L. casei/paracasei* strains were more than 96% identical, whereas identity of *L. rhamnosus* p75 was more than 98% (fig. 1b). Similarity between both groups fall to a 82% identity due to a region of about 160 amino acids at the N-terminal domain which differed in *L. casei/paracasei* and *L. rhamnosus* (fig. 1b). In this region the protein from *L. casei/paracasei* contained a number of repetitions of the Ala-Ala-Ala-Ser sequence and a proline-rich region, whereas in p75 from *L. rhamnosus* several repetitions of the Ala-Ala-Ala-Ser-Gln sequence were found.

p40 and p75 were predicted to have an N-terminal signal peptide recognized by signal peptidase I and were therefore expected to be secreted. A high degree of synteny was observed in the genome regions coding for p40 and p75. However, their genome contexts did not allow hypothesizing a putative function for both proteins.

p40 and p75 Are Secreted in L. casei BL23

In order to determine the likely function and cellular location of p40 and p75 in *L. casei* BL23, mutants in LCABL_00230 (encoding p40) and LCABL_02770 (encoding p75) were constructed in this strain by integration of a plasmid carrying internal fragments of the genes. The obtained mutants BL305 (LCABL_00230::pRV300) and BL306 (LCABL_02770::pRV300) were analyzed for the presence of p40 and p75 in culture supernatants by using sera raised against LGG proteins [Yan et al., 2007] (fig. 2). LGG anti-p40 antibodies reacted against 40- and 75-kDa protein bands present in supernatants of BL23. The 40-kDa band disappeared in BL305 supernatants and the 75-kDa band disappeared in samples from the BL306 strain (fig. 2a). These results confirmed that, as previously reported for LGG, antibodies against p40 cross-reacted to p75 [Yan et al., 2007]. However, no obvious homology was identified between both proteins. LGG anti-p75 antibodies detected a 75-kDa protein which disappeared in the BL306 strain (fig. 2b). In accordance to the mutation present in BL306, faint bands of less than 30 kDa were observed in the supernatant of this strain, which were probably the result of truncated p75 derived from the gene disruption by single cross-over. The results confirmed that, as expected, p40 and p75 were secreted in *L. casei* BL23. A Western blot screening for the presence of p40- and p75-like proteins in the supernatants of several *L. casei/paracasei* strains from different origins revealed the presence of reacting bands in

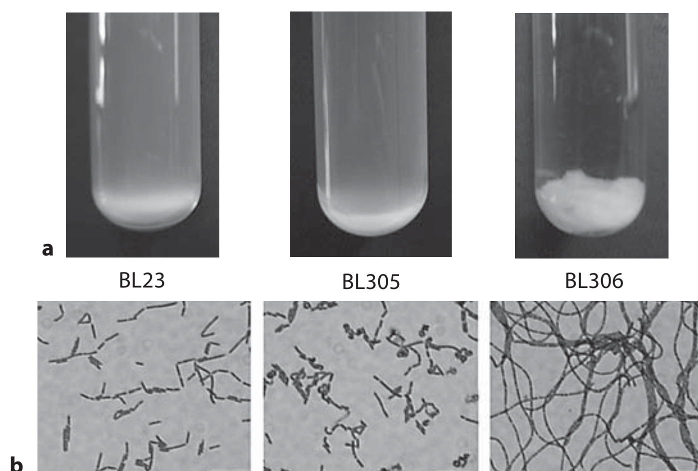

Fig. 4. Growth behavior and cellular morphology of *L. casei* strains lacking p40 and p75. **a** Overnight liquid cultures in MRS medium. **b** Micrographs of wild-type *L. casei* and different mutant strains. The bars represent 10 μm. BL23 (wild-type); BL305 (LCABL_00230::pRV300); BL306 (LCABL_02770::pRV300).

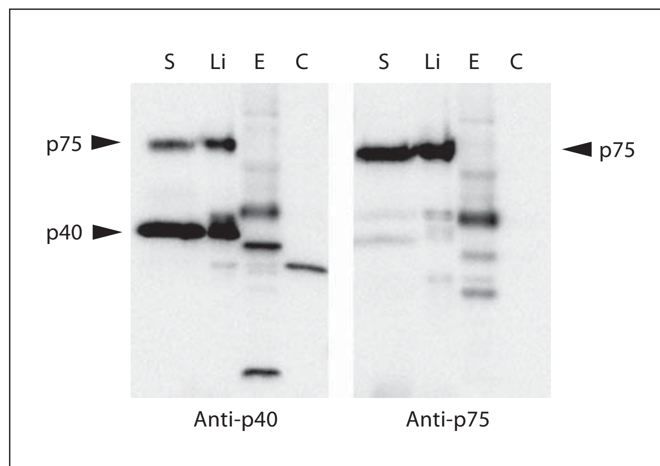

Fig. 5. Extraction of p40 and p75 from the cell surface. A Western blot detection of p40 and p75 in different protein fractions of *L. casei* BL23 is shown. S = Supernatant; Li = proteins extracted with LiCl; E = cell-envelope fraction (cell-wall/membrane fraction); C = cytoplasm. The S lanes contains 25 μl of supernatants, the rest of samples contain 1 μg of protein.

all the strains (fig. 3). This reinforced the idea that these proteins are common in this bacterial group. Small differences in size were detected among the strains, indicating a certain variation in the proteins. In one case, the lower molecular weight detected in p40 could be attributed to changes at the level of protein sequence: compared to the rest of *L. casei/paracasei* sequenced strains, p40 from ATCC334 (BL90) had a deletion of 15 amino acids (fig. 1a), which resulted in a smaller protein of 38 kDa. Only two strains (BL199 and BL212) showed a clear difference in the band pattern, suggesting the presence of different forms of p40 and p75 or proteolysis of the secreted proteins.

p75 Is Involved in Cell Separation

The p40-disrupted mutant (BL305) showed a growth behavior similar to the wild type. However, the p75-disrupted strain (BL306) exhibited an autoaggregating phenotype in liquid medium (fig. 4a). Under the microscope, BL305 displayed some curved forms and shorter bacilli (3.2 ± 0.7 μm) than BL23 (4.1 ± 1.2 μm), whereas BL306 (p75) cells formed very long chains of more than 100 μm (fig. 4b). These long chains were probably responsible for the macroscopic characteristics of the strain in liquid cultures, as they tended to interlace forming cords and balls, and suggested that in this mutant the capacity of cell separation after division was impaired.

p40 and p75 could be extracted from the *L. casei* cell surface by a simple cold water treatment (data not shown) or by extraction with 1.5 M LiCl, whereas they were absent from the cytoplasmic fraction (fig. 5). These results indicated that a fraction of the proteins is attached to the cell surface but not tightly bound to it.

p40 and p75 Are Able to Hydrolyze L. casei *Muropeptides*

Sequence analysis, cellular location and the phenotype of an *L. casei* strain lacking p75 (BL306) suggested that p40 and p75 proteins are enzymes involved in the metabolism of the *L. casei* cell wall. p40 and p75 proteins from *L. casei* BL23 were purified as GST fusions and assayed for the hydrolysis of muropeptides obtained by hydrolysis of *L. casei* cell walls with mutanolysin. Figure 6 shows the HPLC chromatograms of *L. casei* muropeptides after incubation with the purified proteins. Whereas incubation with a GST control did not change the muropeptide profile, GST-p40 and GST-p75 reduced the amount of the most hydrophobic peptides and resulted in the appearance of polar products, possibly smaller peptides, which indicate hydrolytic activity on muropeptides. Therefore, since they are cell-wall-bound muramidases, genes encoding p40 and p75 will hereinafter be called *cmuA* and *cmuB*, respectively.

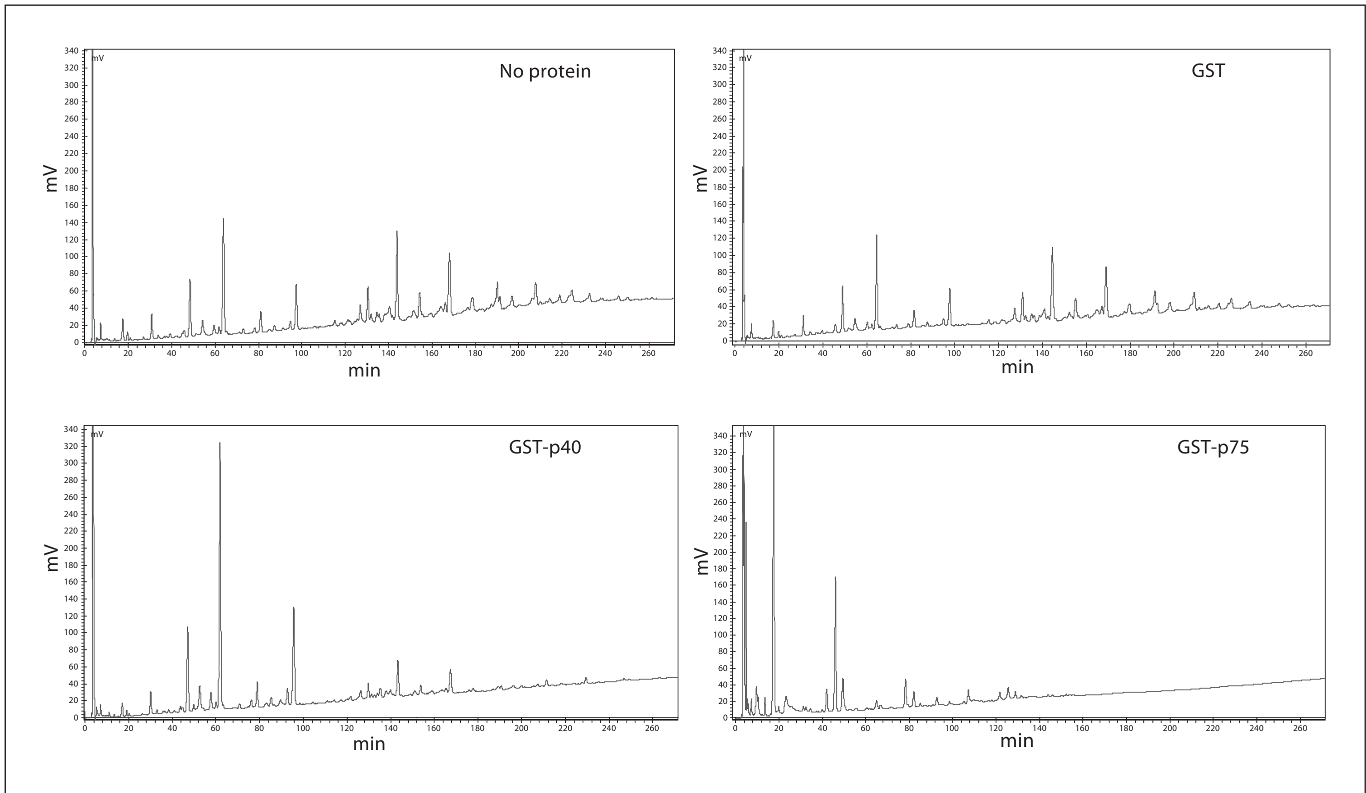

Fig. 6. Hydrolysis of *L. casei* muropeptides by GST-p40 and GST-p75. Muropeptides isolated from *L. casei* BL23 cell walls were incubated without protein or with GST, GST-p40 or GST-p75 and the hydrolysis products were separated by HPLC.

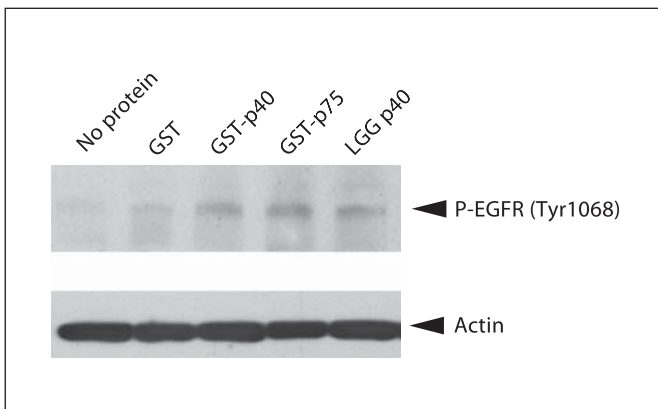

Fig. 7. *L. casei* p40 and p75 stimulates EGFR phosphorylation. Mouse colon fragments were incubated with 10 ng/ml of each protein for 2 h and phosphorylation of EGFR was detected with anti-EGFR-phospho-Tyr1068 antibodies. LGG p40 is p40 from *L. rhamnosus* GG. Anti-actin antibodies were used as a loading control.

p40 and p75 from L. casei *Stimulate Epidermal Growth Factor Receptor Phosphorylation in Mice*

The probiotic activity of LGG p40 has been reported to involve epidermal growth factor receptor (EGFR) and this protein is able to activate the EGFR pathway in an ex vivo colon organ culture model and in vivo [Yan et al., 2010]. Therefore, we tested whether the *L. casei* BL23 p40 and p75 proteins exhibited a similar activity. Figure 7 shows that incubation of mouse colon fragments with either GST-p40 or GST-p75 stimulated EGFR phosphorylation similar to p40 purified from LGG.

L. casei *p40 and p75 Bind to Extracellular Matrix Proteins and Epithelial Cells*

Some surface-associated proteins displaying cell-wall hydrolytic activity from staphylococci, enterococci and *Listeria* are adhesion molecules with extracellular matrix (ECM) and eukaryotic cell binding activity [Milohanic et al., 2001; Teng et al., 2003; Heilmann et al., 2005]. In order to test this possibility, in vitro binding to immobilized mucin, to the ECM proteins fibronectin, fibrinogen and

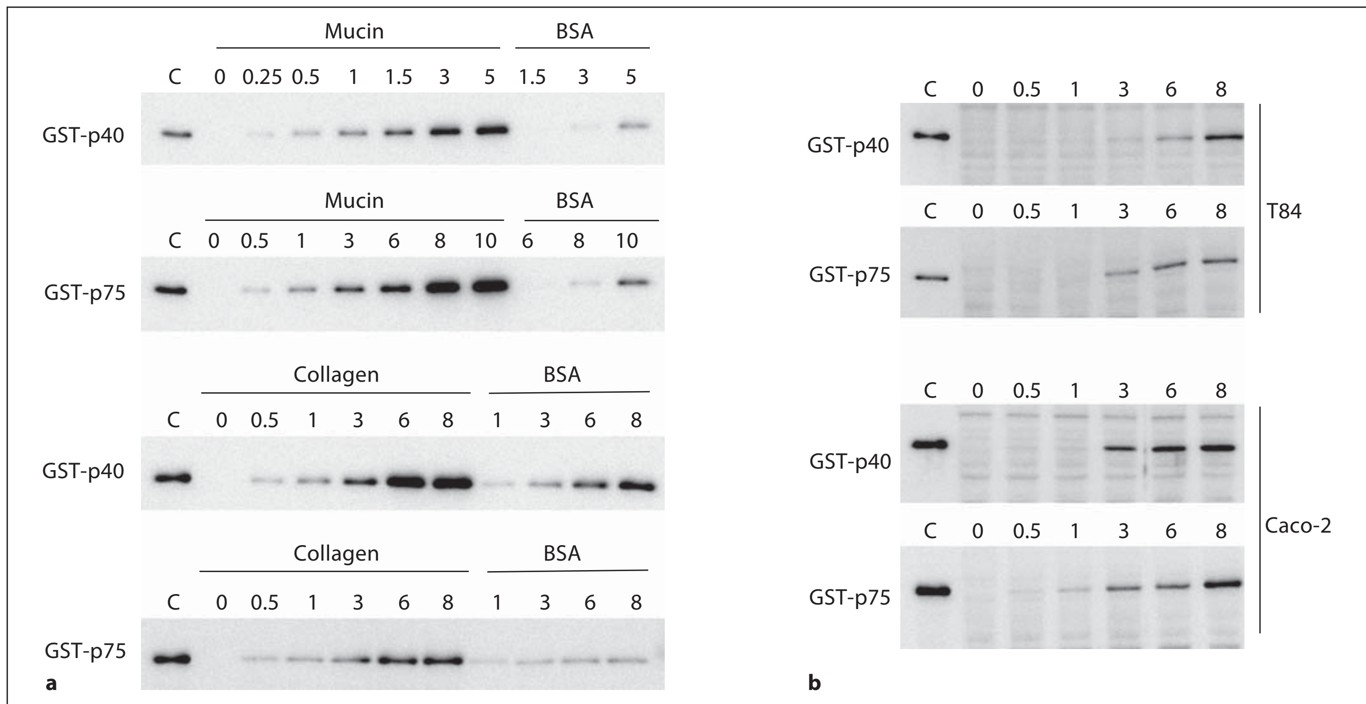

Fig. 8. Binding of *L. casei* p40 and p75 to mucin, collagen and different intestinal epithelial cell lines. **a** Mucin, collagen or BSA were bound to microtiter plates and incubated with different amounts of GST-p40 and GST-p75 (indicated in micrograms by the numbers above each panel). After washing, the bound proteins were released and detected by Western blot. **b** Monolayers of T84 or Caco-2 cells were incubated with different quantities of GST-p40 and GST-p75 (indicated above the panels). After washing, bound proteins were detected by Western blot. Lanes marked as C contain 5 ng of purified proteins as control.

collagen and to T84 and Caco-2 epithelial cells was assayed with GST-p40 and GST-p75. Figure 8a shows that GST-p40 and GST-p75 bound to mucin and collagen in a dose-dependent and saturable manner. No binding was detected for fibronectin or fibrinogen (data not shown). Both fusions bound to BSA much less efficiently. Control experiments in which GST activity was determined in the wells after washing demonstrated that GST by itself did not bind to these molecules (data not shown). Both proteins were also able to bind to T84 and Caco-2 epithelial cells (fig. 8b).

Discussion

Most proteins from probiotic bacteria identified as factors interacting with host cells are non-specialized proteins actually implicated in other bacterial processes. This included the EF-Tu translation elongation factor and the molecular chaperone GroEL from *L. johnsonii* [Granato et al., 2004; Bergonzelli et al., 2006], different glycolytic enzymes from *Lactobacillus plantarum* and *Lactobacillus crispatus* [Hurmalainen et al., 2007; Kinoshita et al., 2008; Ramiah et al., 2008], and the surface-layer protein A from *L. acidophilus* [Konstantinov et al., 2008], involved in epithelial cells and ECM attachment, plasminogen activation and immunoregulation. We showed here that two proteins, p40 and p75, identified as probiotic factors in LGG and also present in members of the *L. casei/paracasei-L. rhamnosus* group, fitted into the group of cell-wall hydrolases, also known as autolysins.

Bacterial autolysins comprise a group of enzymes which display the complete set of activities for peptidoglycan hydrolysis (N-acetyl-muramoyl-L-alanine amidase, N-acetylglucosaminidase, endopeptidase, carboxypeptidase and N-acetylmuramidase) and play a crucial role in bacterial cell growth, shape maintenance and division. p40 and p75 are modular proteins which carry carboxy-terminal catalytic domains with putative amidase and endopeptidase activity, respectively, and N-terminal domains that could be implicated in their interaction with peptidoglycan. In this sense, p75 is an atypical pep-

tidoglycan hydrolase as it lacks any recognized motif involved in peptidoglycan binding in other autolysins (e.g. LysM, GW or SH3 domains [Layec et al., 2008]). On the contrary, a similar N-terminal domain of p40 (COG3883) present in CwlO, a cell-wall endopeptidase from *Bacillus subtilis* [Yamaguchi et al., 2004], has been shown to interact with peptidoglycan. p40 and p75 hydrolytic activity could be demonstrated on *L. casei* BL23 muropeptides and both proteins were secreted and bound to the bacterial surface. The lack of p40 in *L. casei* BL23 only resulted in small changes in cellular morphology. However, p75 was crucial for cell separation. In firmicutes, this function is species-specific and is usually carried out by autolysins carrying CHAP, NLPC/P60 or N-acetylglucosaminidase domains [Layec et al., 2008]. Lack of daughter cell separation activity leads to the formation of long chains of cells or filamentous cells which do not separate but contain fully formed septa [Wuenscher et al., 1993; Gao et al., 2006].

The biological activity of p40 and p75 proteins on intestinal epithelium is still intriguing. Similar to LGG, it has been reported that the presence of p40 and p75 correlated to the capacity of culture supernatants from *L. casei* ATCC393 and *L. casei* ATCC334 to stimulate Akt activation and to inhibit cytokine-induced apoptosis in intestinal epithelial cells. On the contrary, *L. acidophilus* supernatants, which do not contain p40 or p75, did not show such effect [Yan et al., 2007]. LGG p40 and p75 act on intestinal epithelium through an Akt/PI3K-dependent manner mediated by EGFR activation [Yan et al., 2007, 2010; Seth et al., 2008]. *L. casei* BL23 p40 and p75 were also able to stimulate EGFR phosphorylation, suggesting that the activity of p40 and p75 is not strain-specific. Sequence analysis of available genomes and Western blot with bacterial supernatants revealed that p40- and p75-like autolysins are common in *L. casei-paracasei* and *L. rhamnosus* strains, ranging from human to food isolates. It is not known whether the heterogeneity found in these proteins would confer distinct characteristics to the proteins from a particular strain.

In addition to their peptidoglycan hydrolytic activity, some autolysins from bacteria are involved in other processes. Cell-surface-associated autolysins from several pathogens play a role in ECM and epithelial cell attachment and are important virulence factors. SagA from *Enterococcus faecium* and Aaa from *Staphylococcus aureus* are autolysins with a broad spectrum of ECM protein binding, showing capacity to bind to fibrinogen, collagen, fibronectin and laminin [Teng et al., 2003; Heilmann et al., 2005] and the autolytic amidase Ami from *Listeria monocytogenes* participates in binding to human cells through its cell-wall binding domain [Milohanic et al., 2001]. We showed that p40 and p75 from *L. casei* BL23 bind to mucin, collagen and to cultured epithelial cells. *L. casei* BL23 shows low mucin and intestinal epithelial cells adhesion [Muñoz-Provencio et al., 2009, 2010], for which it is not expected that p40 and p75 function as adhesion factors in this strain. However, in agreement to binding experiments, oral administration of FITC-labeled LGG p40 to mice resulted in the detection of p40 on the surface of colon epithelium [Yan et al., 2010]. Whether binding of LGG p40 to the mucosa relates to its effect in mouse intestine remains to be investigated.

It is established that proteins synthesized by probiotics modify host cells responses [Corthesy et al., 2007; Lebeer et al., 2008; Vanderpool et al., 2008]. However, the characterization of these proteins and of their cellular targets is still poor. The work reported here represents new insights in the study of probiotic factors in *L. casei/paracasei-rhamnosus* group, as two cell-wall hydrolases are related to the described functional properties of these bacteria. The activity of p40 and p75 could reside in either the N-terminal domains or the catalytic C-terminal domains. The construction of different truncated versions of the proteins and point mutation derivatives with abolished catalytic activity will help to answer this question.

Experimental Procedures

Bacterial Strains and Growth Conditions

Lactobacillus strains are listed in table 1 and they were grown in MRS medium (Difco) at 37°C under static conditions. *L. casei* mutant strains obtained by gene disruption were maintained in MRS supplemented with erythromycin at 5 μg/ml. *Escherichia coli* DH5α was used as a host for cloning and *E. coli* BL21(DE3)-[pLysS] was used for protein expression and purification. They were grown in LB medium at 37°C under agitation. Recombinant plasmids in *E. coli* were selected with ampicillin at 100 μg/ml and chloramphenicol at 20 μg/ml. Solid medium was prepared by adding 1.8% (w/v) agar.

Construction of L. casei *Mutant Strains*

Internal fragments of LCABL_00230 (encoding p40) and LCABL_02770 (encoding p75) genes were obtained by PCR with primer pairs 5′-TTGAAGCTTAAAAGAATGTCACAGC/5′-TGAGGTACCAGGTGCACTG and 5′-CCTGGTACCCAG-ACAGCAA/5′-GCAGAATTCGGCGTTGG, respectively, using chromosomal DNA from *L. casei* BL23 as amplification template (restriction sites introduced for cloning are underlined). The fragments were cloned into the integrative vector pRV300 [Leloup et al., 1997] digested with *Kpn*I/*Hin*dIII and *Kpn*I/*Eco*RI, respectively. *L. casei* electrocompetent cells were obtained as described [Posno et al., 1991], transformed with these constructs and plated on

Table 1. *L. casei/paracasei* strains used in this study

Strain	Selected characteristics	Origin
BL6	type strain	ATCC[a] 393
BL23	laboratory strain; sequenced genome [Acedo-Felix and Perez-Martinez, 2003]	CECT[b] 5275
BL32	cheese isolate	CECT4040
BL82	sour milk isolate	ATCC25598
BL83	cheese isolate	CECT4043
BL87	oral cavity isolate	ATCC11578
BL90	cheese isolate; sequenced genome	ATCC334
BL91	dental caries isolate	ATCC4545
BL101	isolated from commercial probiotic drink	laboratory stock
BL106	isolated from commercial probiotic drink	laboratory stock
BL193	isolated from commercial probiotic drink	laboratory stock
BL199	exopolysaccharide producer [Mozzi et al., 1996]	CRL[c] 87
BL208	human intestinal isolate	laboratory stock
BL212	dry cured sausage isolate [Fadda et al., 1998]	CRL686
BL227	commercial probiotic	laboratory stock
BL229	commercial probiotic	laboratory stock
BL305	BL23 *cmuA*(LCABL_00230)::pRV300, ery^r	this work
BL306	BL23 *cmuB*(LCABL_02770)::pRV300, ery^r	this work

ery^r = Erythromycin resistant.
[a] American Type Culture Collection. [b] Colección Española de Cultivos Tipo. [c] Centro de Referencia para Lactobacilos.

MRS plates supplemented with erythromycin. Integration of the suicide vector at the correct locus was checked by PCR on chromosomal DNA isolated from erythromycin-resistant colonies using appropriate oligonucleotides. Plasmid integrations resulted in strains with 104 and 126 amino acid deletions at the C-terminus of p40 and p75, respectively.

Preparation of Cellular Fractions

L. casei bacterial cells were grown in 50 ml of MRS to late exponential phase and washed twice with PBS (pH 7.4). In order to isolate surface proteins, the pellet was resuspended in a solution containing 10 mM Tris-HCl pH 8 and 1.5 M LiCl and incubated at 4°C for 1 h. Bacteria were recovered by centrifugation at 6,000 *g* for 10 min and proteins in the supernatant were precipitated by adding trichloroacetic acid to 10% final concentration. After incubation at 4°C for 1 h, precipitated proteins were recovered by centrifugation at 10,000 *g* for 20 min, washed with cold 96% ethanol and resuspended in 7 M urea. The bacterial pellet was resuspended in PBS, disrupted with glass beads and unbroken cells were discarded by three centrifugations at 6,000 *g* for 5 min. The supernatant was then centrifuged at 22,000 *g* for 20 min at 4°C. The soluble fraction was retained as the cytoplasm fraction, whereas the pellet was washed 3 times at 22,000 *g* for 15 min with 50 mM Tris-HCl pH 8 + 0.5 M NaCl and retained as the cell-envelope fraction (cell-wall/membrane fragments).

Western Blotting

Samples were separated in 10% SDS-PAGE gels and electrotransferred to Hybond-ECL membranes (GE Healthcare). Membranes were blocked in a 5% (w/v) non-fat dry milk solution in 50 mM Tris-HCl pH 7.6, 150 mM NaCl (TBS) containing 0.1% (v/v) Tween-20 and incubated with a 1:10,000 dilution of rabbit anti-p40 or anti-p75 serum [Yan et al., 2007] for 1 h. After three 15-min washes with TBS + 0.1% Tween-20, bound antibodies were detected with a 1:20,000 dilution of goat anti-rabbit HRP-conjugated antibody (GE Healthcare) and the ECL-advance chemiluminescent reagents as described by the supplier (GE Healthcare). Light emission from the blots was quantified with a LAS-1000 image analysis system (Fuji, Tokyo, Japan).

Protein Purification

L. casei BL23 p40 and p75 proteins were purified as glutathione-S-transferase (GST) fusion proteins after expression in *E. coli*. LCABL_00230 was amplified with the primer pair 5′-GTTGGATCCGATACAAGCGACAG/5′-GTGGGTACC-TTACCGGTGGATATAA and *L. casei* BL23 chromosomal DNA. The obtained fragment was digested with *Bam*HI and *Kpn*I and cloned into the pGEX2t vector (GE Healthcare) digested with the same enzymes. LCABL_02770 was amplified with the primer pair 5′-GTTAGATCTTCAACGGGGACA/5′-TAGGGTACCTT-ATAGTGAAGGACG. The PCR product was digested with *Bgl*II and *Kpn*I and cloned into pGEX2t. Both recombinant plasmids were sequenced and transformed into *E. coli* BL21(DE3)[pLysS]. Recombinant bacteria were grown in 500 ml of LB supplemented with ampicillin and chloramphenicol at 37°C. When bacterial cells reached an $OD_{600\ nm}$ of 0.4, 1 mM IPTG was added and growth continued for 3 h. The bacterial pellet was recovered by centrifugation and bacteria were broken by sonication. GST-fu-

sion proteins were purified in glutathione-Sepharose columns (1 ml bed volume) as recommended by the manufacturer (GE Healthcare). Purified proteins were dialyzed against a buffer containing 50 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, and 15% (v/v) glycerol at 4°C and aliquots were stored at –80°C.

HPLC Analysis of the Hydrolysis of L. casei *Muropeptides*

In order to isolate *L. casei* BL23 muropeptides, cell-wall peptidoglycan was prepared as described previously [Meyrand et al., 2007]. Briefly, a 500-ml culture ($OD_{600\,nm}$ of 0.4, 2.6 × 10^8 cfu/ml) was chilled on ice and cells were harvested by centrifugation. Cells were boiled in 20 ml of deionized water for 10 min. After centrifugation, they were resuspended and boiled in 2 ml of 5% SDS for 25 min. Insoluble material was recovered and boiled again in 4% SDS for 15 min. The resulting cell-wall pellet was washed at least 6 times with hot deionized water to eliminate the SDS. Proteins were removed by treatment with 2 mg/ml of pronase (90 min, 60°C) and 200 μg/ml trypsin (16 h, 37°C). Cell walls were collected by centrifugation (22,000 *g*, 10 min), washed once in deionized water, resuspended in 800 μl of hydrofluoric acid (48% v/v solution) and incubated at 4°C for 24 h. The resulting insoluble material was centrifuged (22,000 *g*, 4°C) and washed twice with 250 mM Tris-HCl pH 8 and at least 4 times with deionized water.

Purified peptidoglycan was digested with mutanolysin (2,500 U/ml; Sigma) for 19 h at 37°C under end-over-end agitation. The enzyme was inactivated by boiling the samples for 3 min and the soluble muropeptides were reduced with sodium borohydride [Dougherty, 1985]. To determine hydrolytic activity of recombinant GST-p40 and GST-p75 on soluble muropeptides, pH of the muropeptide solution was adjusted to 7 and 50 μg of purified protein was added to 100 μl of muropeptides. This mixture was incubated for 20 h at 37°C and then separated by reverse-phase HPLC as previously described [Atrih et al., 1999] using a Supelcosil LC-18-DB column at 52°C (4.6 × 150 mm, 5 μm particle size; Sulpeco) and a Jasco PU-2080Plus system with detection at 202 nm.

Ex vivo Mouse Colon Organ Culture

Colon organ culture was performed as described before [Yan et al., 2007]. Briefly, colon explants were isolated from 6- to 8-week-old C57BL/6 mice, washed with sterile PBS and DMEM (Gibco) media and then cut into 4 × 4-mm pieces. These colon pieces were cultured on Netwell™ inserts (Corning Life Sciences Inc.) in DMEM (Gibco) containing 0.5% fetal bovine serum and incubated at 37°C with 5% CO_2 for 2 h before treatment. Proteins (GST fusions and p40 isolated from LGG supernatant [Yan et al., 2007]) were added at a final concentration of 10 ng/ml and explants were further incubated for 2 h. At the end of the experiment, the colonic mucosa was scraped into homogenization buffer and tissue lysed [Polk, 1994]. The protein concentration was determined using the DC protein assay (Bio-Rad Laboratories). Equal amounts of cellular lysate proteins were mixed with Laemmli sample buffer and separated by SDS-polyacrylamide gel electrophoresis for Western blot analysis with anti-EGFR-phospho-Tyr1068 (Cell Signaling Technology) and anti-actin (Upstate) polyclonal antibodies.

Binding to ECM Proteins and Epithelial Cells

Binding of GST-p40 and GST-p75 to immobilized fibronectin (human plasma; Sigma), fibrinogen (fraction I from pig plasma; Sigma), collagen (type I; Roche) and mucin (porcin stomach; Sigma) was assayed in 96-well immunoplates. Polysorp plates (Nunc) were covered overnight at 4°C with 10 μg/ml fibronectin or fibrinogen in 50 mM carbonate/bicarbonate buffer pH 9.6, whereas Maxisorp plates (Nunc) were used for immobilization of 50 μg/ml collagen in PBS pH 5.5 or 500 μg/ml mucin in 50 mM carbonate/bicarbonate buffer pH 9.6. The plates were washed 3 times with PBS and blocked with 1% BSA for at least 1 h at 37°C. 100 μl of GST, GST-p40 and GST-p75 at different concentrations were added to each well and incubated for 1 h at 37°C. The wells were washed 8 times with PBS containing 0.05% Tween-20 in order to eliminate unbound proteins. Bound proteins were solubilized by addition of 40 μl of Laemmli buffer. 5-μl aliquots were subjected to SDS-PAGE and the amount of bound GST-p40 and GST-p75 was detected by Western blot. In order to assess unspecific binding of GST to ECM components, GST, GST-p40 and GST-p75 were incubated with immobilized ECM components as described above. After washing of unbound proteins, GST activities of bound proteins were determined using the substrate 1-chloro-2,4-dinitrobenzene together with reduced glutathione and the reaction was monitored by measuring the absorbance at 340 nm.

For binding assays to intestinal epithelial cells, Caco-2 cells were seeded in 96-well plates at 25,000 cells/well in DMEM (Gibco) medium supplemented with 10% fetal bovine serum, 10 mM Hepes, 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin (Gibco)). T84 cells were seeded in 96-well plates at 65,000 cells/well and grown in DMEM/F-12 medium (Gibco) containing 10% fetal bovine serum and antibiotics. Cells were used for experiments after 16–18 days (Caco-2) or 11–13 days (T84) of culture. Different concentrations of GST-p40 and GST-p75 were diluted in the respective cell culture medium and incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After 8 washing steps with PBS, bound GST-p40 and GST-p75 were eluted with Laemmli buffer and detected by Western blot as described above.

Acknowledgements

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