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Identification and Functional Characterization of the *Lactococcus lactis* CodY-Regulated Branched-Chain Amino Acid Permease BcaP (CtrA)

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Transcriptome analyses have previously revealed that a gene encoding the putative amino acid transporter CtrA (YhdG) is one of the major targets of the pleiotropic regulator CodY in *Lactococcus lactis* and *Bacillus subtilis*. The role of *ctrA* in *L. lactis* was further investigated with respect to both transport activity as well as CodY-mediated regulation. CtrA is required for optimal growth in media containing free amino acids as the only amino acid source. Amino acid transport studies showed that *ctrA* encodes a secondary amino acid transport system that is specific for branched-chain amino acids (BCAAs) (isoleucine, leucine, and valine) and methionine, which is in disagreement with its previously proposed function (a cationic amino acid transporter), which was assigned based on homology. We propose to rename CtrA BcaP, for branched-chain amino acid permease. BcaP is a member of a group of conserved transport systems, as homologs are widely distributed among gram-positive bacteria. Deletion of *bcaP* resulted in the loss of most of the BCAA uptake activity of *L. lactis*, indicating that BcaP is the major BCAA carrier of this organism. Deletion of *bcaP* together with a second (putative) BCAA permease, encoded by *brnQ*, further reduced the viability of the strain. DNA microarray analysis showed that deletion of *bcaP* predominantly affects genes belonging to the regulons of the transcriptional regulator CodY, which is involved in global nitrogen metabolism and needs BCAAs for its activation, and of CmbR, which is involved in sulfur amino acid metabolism.

CodY is a well-studied transcriptional regulator that was first identified as the nutritional repressor of the dipeptide permease operon in *Bacillus subtilis* (55). Functional homologs of CodY are present in several gram-positive bacteria in which the protein is involved in the regulation of a wide array of genes (44, 56). A recent study has unraveled the genome-wide effects of CodY on gene expression of *Lactococcus lactis* (6). In this lactic acid bacterium (LAB), the majority of the CodY-regulated genes are involved in the proteolytic system (5, 18, 19). Proteolysis is essential to *L. lactis*, as it allows this organism to utilize the caseins present in milk as a source of essential amino acids (32). Efficient casein utilization requires the activities of an extracellular proteinase and various peptide transporters and intracellularly located peptidases (reviewed in reference 28). In addition to the transport systems for (oligo)peptides, *L. lactis* contains multiple (putative) permeases that facilitate the internalization of free amino acids (4). At least 10 of these systems, differing with respect to their specificity, have been characterized biochemically in various LAB to date (29), but most of the encoding genes have not yet been identified (33).

Besides the prominent role of CodY in coordinating expression of the genes that constitute the proteolytic system of *L. lactis*, transcriptome analysis of a lactococcal *codY* mutant revealed that several transcriptional units involved in the metabolism of certain amino acids are CodY controlled as well (6). Particularly, transcript levels of the glutamate, histidine, and branched-chain amino acid (BCAA) biosynthetic operons (*gltDB*, *his*, and *leu-ilv*, respectively) were strongly elevated upon deletion of *codY*. Although *L. lactis* MG1363 is auxotrophic for histidine and BCAAs (49), due to frameshifts and small deletions in the coding regions of their biosynthetic operons, expression of the genes is apparently still tightly regulated.

The finding that CodY also regulates the transcription of genes involved in the metabolism of amino acids other than BCAAs was surprising, since BCAAs are solely responsible for the activation of lactococcal CodY (5, 19, 48) and thus have a central role in CodY-mediated regulation. In both *L. lactis* and *B. subtilis*, BCAAs modulate the activity of CodY by increasing the affinity of the regulator for its operator sites. In *B. subtilis*, an additional level of regulation of CodY activity is provided by GTP, a marker of the energy state of the cell, which stimulates CodY activity independently of BCAAs (50, 54). CodY is most active in rapidly growing *L. lactis* cells, when BCAAs are abundant. When intracellular levels drop, repression by CodY is relieved, which results in the derepression of the proteolytic system and a concomitant increase in the capacity to utilize milk proteins.

L. lactis preferably uses oligopeptides as a source of amino acids during growth in milk (25). Although free amino acids are rather scarce in milk (43), they probably affect intracellular amino acid pools via the activity of amino acid uptake systems. These uptake systems, therefore, could be important for CodY-mediated regulation. Indeed, one of the newly identified members of the lactococcal CodY regulon encodes a putative amino acid transporter (6). This gene, *ctrA*, was found to be one of the main targets of CodY in DNA microarray experi-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Source or reference
L. lactis strains		
MG1363	Lac^{-} Prt^{-}; plasmid-free derivative of NCDO712	13
NZ9000	MG1363 *pepN*::*nisRK*	31
NZ9700	Nisin-producing transconjugant of MG1363 containing Tn*5276*	30
MG*brnQ*	MG1363 derivate, chromosomal deletion of *brnQ*	This work
MG*bcaPbrnQ*	MG1363 derivate, chromosomal deletion of *bcaP* and *brnQ*	This work
MG*bcaP*	MG1363 derivate, chromosomal deletion of *bcaP*	This work
Plasmids		
pNZ8048	Cm^{r}; expression vector carrying the nisin-inducible P_{nisA}	26
pNZ9530	Ery^{r}; *nisRK* cloned in pIL252; constitutive expression of *nisRK*	26
pVE6007	Cm^{r}; temperature-sensitive replication derivate of pWV01	40
pORI280	Ery^{r} *ori*$^{+}$ RepA^{-}; *lacZ* expressed constitutively from P32 promoter	38
pNG*bcaP*	pNG8048 containing *bcaP* gene	This work
pNG*bcaP-H6*	pNG8048 containing *bcaP-his6* of *L. lactis* MG1363 behind P_{nisA}	This work
pORIΔ*bcaP*	Ery^{r} LacZ^{+}; pORI280 containing *bcaP* deletion construct	This work
pORIΔ*brnQ*	Ery^{r} LacZ^{+}; pORI280 containing *brnQ* deletion construct	This work
pORIΔ*bcaPbrnQ*	Ery^{r} LacZ^{+}; pORI280 containing *bcaP* and *brnQ* deletion construct	This work

ments. Transcriptional regulation of *ctrA* by CodY was confirmed by expression studies in which the upstream region of *ctrA* was fused to the reporter gene *lacZ*; the amount of *lacZ* mRNA was increased almost 10-fold in a *codY* deletion strain grown in nitrogen-rich medium. In addition, these experiments showed that the mRNA of the reporter was highly abundant in the mutant strain, indicating that *ctrA* is preceded by a strong promoter. The upstream region of *ctrA* contains three copies of a conserved nucleotide stretch, termed the CodY box, that has recently been shown to serve as an operator site for CodY (6, 19a). Electrophoretic mobility shift assays and DNase I footprinting analyses revealed that CodY directly interacts with this sequence in the upstream region of *ctrA* (6). *B. subtilis* contains a homolog of CtrA (i.e., YhdG). Like lactococcal *ctrA*, transcription of *yhdG* is strongly affected by CodY, as it was derepressed 116-fold in a *codY* deletion strain in a DNA microarray analysis (44).

The *ctrA* gene product (a predicted cationic amino acid transporter) was designated as such because it contains domains that are conserved between the cationic amino acid permeases LysP, PotE, and AnsP, which transport lysine, putrescine/ornithine, and γ-aminobutyrate, respectively (4, 41). Considering the tight regulation of *ctrA* in both *L. lactis* and *B. subtilis* and the absence of an apparent link between the metabolism of cationic amino acids and CodY regulation, we wondered which role *ctrA* serves in the physiology of *L. lactis*. Here, we report on the cloning, functional expression, and characterization of *ctrA*. We show that CtrA, in disagreement with its predicted function, is a transporter of BCAAs and demonstrate its importance for CodY-mediated regulation. Based on its newly identified function, we propose to rename CtrA BcaP, for branched-chain amino acid permease.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* was grown in twofold-diluted M17 broth (61) supplemented with 0.5% glucose (½GM17) at 30°C or on ½GM17 solidified with 1.5% agar. When appropriate, 5 μg ml^{-1} of erythromycin and/or chloramphenicol (both from Sigma Chemical Co., St. Louis, MO) was added. Alternatively, cells were grown in a chemically defined medium (CDM), prepared as described previously (42), supplemented with 1% Casitone (Difco Laboratories, Detroit, MI) or with specific dipeptides where indicated. Growth in 96-well plates incubated at 30°C was monitored using a GENios microtiter plate reader (Tecan, Grödig, Austria). The A_{595} of the cultures was measured every 30 min following 15 s of shaking.

DNA manipulation, molecular cloning, and transformation. Routine DNA manipulations were performed as described previously (51). Total chromosomal DNA from *L. lactis* was extracted as described previously (39). Minipreparations of plasmid DNA from *L. Lactis* were made using the High Pure plasmid isolation kit from Roche Molecular Biochemicals (Mannheim, Germany). Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals. PCR amplifications were carried out using *Pwo* DNA polymerase (Roche Molecular Biochemicals) and oligonucleotides listed in Table 2. PCR products were purified with the High Pure PCR product purification kit (Roche Molecular Biochemicals). Electrotransformation of *L. lactis* was performed using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) as described previously (22).

Construction of *L. lactis* deletion mutant strains. DNA fragments containing approximately 600 bp of the flanking regions of the *bcaP* gene of *L. lactis* MG1363 were obtained by PCR using the oligonucleotide pairs ctrA-P1/ctrA-P2 and ctrA-P3/ctrA-P4 (Table 2), cut with the appropriate restriction endonucleases, and ligated into plasmid pORI280, a conditionally replicating vector (38). The resulting plasmid, pORIΔ*bcaP*, was introduced into *L. lactis* MG1363 by electroporation together with the helper plasmid pVE6007. Following chromosomal integration of pORIΔ*bcaP*, cells were grown for about 60 generations under nonselective conditions after which a clone, designated MG*bcaP*, in which *bcaP* was deleted was obtained. The *bcaP* mutation was confirmed by PCR and

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′–3′)[a]
ctrA-P1	GCTCTAGACTAGAATTAGGACATATATCAC
ctrA-P2	CGCGGATCCCTCATAAATCCCATAATAAAT CCTC
ctrA-P3	CGCGGATCCTGGTTCCTTATTGGAATTGCG
ctrA-P4	CCGGAATTCAACGGCTGGTACGATACGAAC
brnQ-P1	GCTCTAGACATTAGTCCAAATGGCGATACC
brnQ-P2	CGCGGATCCGATAGTCTTTACCAGCTAGTTTC
brnQ-P3	CGCGGATCCGAGCTTCTAATATTTAGGAGCTC
brnQ-P4	CCGGAATTCCATCTCGGTTTTAACGTCTGAAC
ctrA-N	CTAGACCACCATGGGATTTATGAGAAAAGCC
ctrA-C	CTAGTCTAGACGTCTTATTTCTTTTTGCGACG
ctrA-CH6	CTAGTCTAGATTAGTGATGGTGATGGTGATG TTTCTTTTTGCGACGATTTCCATAA

[a] Restriction enzyme sites are underlined.

Southern blot analysis (51) using the ECL direct nucleic acid labeling system (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). *L. lactis* MG1363 strains in which either *brnQ* or both *brnQ* and *bcaP* are deleted (MG*brnQ* and MG*bcaPbrnQ*, respectively) were obtained using the same double-crossover strategy with primer combinations brnQ-P1/brnQ-P2 and brnQ-P3/brnQ-P4 for the deletion of *brnQ* (Table 2).

Overproduction and immunodetection of native and histidine-tagged BcaP. Full-length *bcaP* of *L. lactis* MG1363 was amplified from the chromosome by PCR using oligonucleotides ctrA-N and ctrA-C (Table 2), which introduced 5′ NcoI and 3′ XbaI restriction enzyme sites, respectively. Alternatively, a sequence encoding a hexahistidine tag was fused to the 3′ end of *bcaP* using oligonucleotides ctrA-N and ctrA-CH6. The purified PCR products were digested with NcoI and XbaI and ligated downstream of the nisin-inducible promoter P_{nisA} using the corresponding sites in pNG8048, resulting in pNG*bcaP* and pNG*bcaP-H6*, respectively. The plasmids were introduced into *L. lactis* strain NZ9000, MG1363, or MG*bcaP*, together with plasmid pNZ9530 (26) in the latter two strains, to enable nisin-induced production of BcaP or histidine-tagged BcaP (BcaP-H6), as described previously (7).

Production of BcaP-H6 was detected by Western hybridization. To this end, samples were taken from cultures growing in ½GM17 containing the appropriate antibiotics. After 2 h of induction with a 1,000-fold-diluted supernatant of a culture of the nisin-producing *L. lactis* strain NZ9700 (30) grown overnight, approximately 10^8 cells were harvested by centrifugation, resuspended in 100 μl Birnboim solution A (51) containing 1 mg/ml lysozyme (Merck KGaA, Darmstadt, Germany), and incubated for 15 min at 48°C. Proteins were separated on 10% polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (36) and transferred onto polyvinylidene difluoride membranes (Roche Molecular Biochemicals). BcaP-H6 was visualized with specific anti-His antibodies and peroxidase-anti-mouse conjugates (both from Amersham Biosciences) according to the supplier's instructions.

Amino acid transport assays. *L. lactis* MG1363 (pNG*bcaP*; pNZ9530), *L. lactis* MG1363, or *L. lactis* MG*bcaP*, with the latter two harboring plasmids pNG8048 and pNZ9530, was grown at 30°C in 50 ml of ½GM17. At an optical density at 600 nm (OD_{600}) of approximately 0.4, 50-μl volumes of supernatant of the nisin-producing strain *L. lactis* NZ9700 (30) were added to the cultures to induce BcaP production. At an OD_{600} of approximately 1.0, cells were harvested by centrifugation at 6,300 × *g* at 4°C. Cell pellets were washed with 25 ml of ice-cold CDM, concentrated to an OD_{600} of 10.0 in CDM lacking amino acids, and stored on ice until use. Cells were energized prior to transport assays by 5 min of incubation at 30°C in the presence of 0.5% glucose. Subsequently, 100 μl of cell suspension was mixed with 100 μl of CDM containing 500 μM of the unlabeled amino acid of interest and 0.05 μCi of the same amino acid in a ^{14}C- or ^{35}S-labeled form (Amersham Biosciences). For competition experiments, a 10-fold excess of unlabeled amino acid was added. Mixtures were incubated at 30°C for various time intervals while being stirred. The reactions were stopped by the addition of 2 ml of ice-cold 0.1 M LiCl, followed by filtration through 0.45-μm-pore-size nitrocellulose filters (Schleicher & Schuell GmbH, Dassel, Germany). Reaction tubes and filters were washed with another 2 ml of ice-cold 0.1 M LiCl. Subsequently, the filters were transferred to vials containing 2 ml of scintillation fluid (Packard BioScience, Groningen, The Netherlands), and radioactivity was determined using a Packard TriCarb 2000 CA liquid scintillation analyzer (Packard Instruments, Meriden, CT).

DNA microarray analysis. DNA microarray experiments were performed essentially as described previously (6). Briefly, RNA was isolated from four separately grown cultures of *L. lactis* MG1363 and *L. lactis* MG*bcaP*. Subsequently, single-strand reverse transcription (amplification) and indirect labeling of total RNA with either Cy3 or Cy5 dye (Amersham Biosciences) were performed in duplicate. Labeled cDNA samples were hybridized onto slides containing amplicons representing 2,450 open reading frames (ORFs) of *L. lactis* MG1363 spotted in duplicate. After hybridization, slides were washed and scanned. Slide data were processed and normalized as described previously (62), yielding average ratios of gene expression levels of the mutant to those of the wild-type strain. Expression of a gene was considered to be significantly altered when its ratio of expression in the mutant compared to the wild type was >1.5 or <0.67 and had a CyberT Bayesian *P* value of <0.001. All DNA microarray data, including the slide images and raw data, obtained in this study are available online (http://molgen.biol.rug.nl/publication/bcaP_data/).

Nucleotide sequence accession numbers. The *L. lactis* MG1363 *bcaP* and *brnQ* nucleotide sequences have been assigned GenBank accession numbers DQ377686 and DQ377687, respectively.

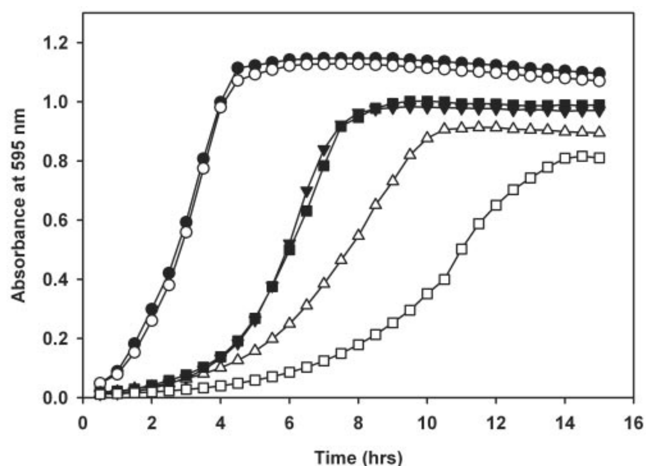

FIG. 1. Growth curves of *L. lactis* MG1363 and its *bcaP* deletion mutant. Growth rates of *L. lactis* MG1363 and MG*bcaP* in CDM supplemented with Casitone (closed and open circles, respectively) are shown. *L. lactis* MG1363, MG*bcaP*, and MG*bcaP* overproducing BcaP were grown in CDM containing free amino acids as the sole source of amino acids (closed triangles, open triangles, and closed squares, respectively). Alternatively, *L. lactis* MG*bcaP* was grown in CDM^{75}, a medium in which the amino acid concentration was lowered to 75% of that in CDM (open squares). Cells were grown at 30°C, and the absorbance of the culture at 595 nm was measured every 30 min. Shown are the means of at least two independent experiments.

RESULTS

Characterization of the *L. lactis* MG1363 *bcaP* (*ctrA*) locus. A gene encoding the putative amino acid transporter CtrA was previously identified in a genome-wide screen for CodY targets in *L. lactis* MG1363 (6). The results presented below show that CtrA is in fact a branched-chain amino acid permease. Therefore, we propose to rename the gene and protein *bcaP* and BcaP, respectively. The 1,398-nucleotide-containing *bcaP* ORF encodes a protein of 465 amino acid residues with a predicted molecular mass of 49.6 kDa and an isoelectric point of 9.91. The *bcaP* ORF starts at an AUG codon following a proper lactococcal ribosome-binding site (GAGGA). Regions that could serve as promoter elements are present upstream of the start codon. These putative −35 (TTGACA) and −10 (TAA AAT) sequences are separated by 17 bp and are likely to constitute the promoter, as DNA fragments comprising this region can readily drive transcription (6). In addition, three copies of a CodY-responsive element (CodY box) can be discerned in the regulatory region of *bcaP*. Downstream of *bcaP*, a palindromic sequence could form a stem-loop structure with a predicted free energy value of −13.8 kcal/mol that could serve as a *rho*-independent transcriptional terminator.

***bcaP* is required for optimal growth of *L. lactis* in media containing free amino acids as the sole amino acid source.** As transcription of *bcaP* is tightly regulated by the global regulator CodY in both *L. lactis* and *B. subtilis* and the protein is conserved among several gram-positive bacteria, we wondered which biological function it serves. To address this question, the entire *bcaP* gene was removed from the chromosome of *L. lactis* MG1363 by double-crossover recombination. The mutated strain, MG*bcaP*, was examined for its ability to grow in media with different amino acid or peptide contents (Fig. 1).

Growth of strains MG1363 and MG*bcaP* was similar in CDM supplemented with Casitone. This nitrogen source consists of a tryptic digest of milk caseins and contains a wide variety of peptides of different lengths and compositions as well as free amino acids. The absence of a growth difference between the strains in this medium indicates that *bcaP* does not serve any apparent physiological function during growth under nitrogen-rich conditions. MG*bcaP* behaved differently when growth was monitored in basal CDM, which contains a mixture of all 20 amino acids in the free form in concentrations ranging from 0.2 to 5 mM but lacks peptides as amino acid source. MG*bcaP* reached a lower final cell density, and the maximum growth rate was significantly reduced (almost twofold) compared to that of the wild-type strain (Fig. 1). When the amino acid concentration was lowered to 75% of that in CDM (CDM^{75}), growth of strain MG*bcaP* was hampered even more (Fig. 1), while the wild-type strain showed normal growth rates and final cell densities (data not shown). These results show that the presence of *bcaP* is required for optimal growth of *L. lactis* in media containing limiting amounts of amino acids.

Phenotypic complementation of the *bcaP* gene deletion. In order to rule out the possibility that the observed differences in growth were due to polar effects on the transcription of neighboring genes that could have been introduced through construction of the mutant, *L. lactis* MG*bcaP* was complemented by full-length *bcaP*. To this end, the *bcaP* gene was placed under the control of a nisin-inducible promoter in plasmid pNG8048 and introduced into the *bcaP* mutant strain. However, no protein band corresponding to BcaP could be detected by SDS-PAGE after induction of BcaP synthesis with nisin. Therefore, a histidine tag was fused to the 3′ terminus of *bcaP*, and proper BcaP synthesis was verified by SDS-PAGE followed by Western hybridization. A protein band specific for BcaP-H6 could indeed be detected by Western analysis upon induction of BcaP-H6 synthesis (data not shown). The size of the protein in this band corresponded to a lower molecular mass than the predicted 50.4 kDa for BcaP-H6, but it has been reported previously that membrane proteins can migrate relatively fast using SDS-PAGE (58). As can be seen in Fig. 1, normal growth was restored when native BcaP synthesis was induced in MG*bcaP* (pNG*bcaP*) grown in CDM containing the free amino acid mixture. These results show that *bcaP* can be provided in *trans* to complement MG*bcaP* and that polar effects of the *bcaP* mutation on growth are absent.

A *bcaP* mutation is bypassed by the addition of the BCAAs or BCAA-containing dipeptides to the growth medium. The experiments described above indicated that *bcaP* encodes a functional protein that is required for optimal growth in the presence of free amino acids. To find out whether BcaP facilitates amino acid transport, as assumed from BLAST results and, if so, to gain insight in its substrate specificity, growth experiments with MG*bcaP* were carried out in media with different amino acid concentrations. Reasoning that the absence of BcaP in the mutant strain would likely result in suboptimal intracellular levels of its substrate(s), the addition of an excess of these amino acids is expected to restore normal growth, provided that an alternative internalization pathway (e.g., another carrier) is present. Based on homology, BcaP is predicted to transport cationic amino acids, hence its original name, CtrA (4). However, no stimulatory effect on the growth of MG*bcaP* was observed when the medium was supplemented with lysine, arginine, putrescine, ornithine, or γ-aminobutyrate at a concentration of 5 mM of each of these compounds (data not shown). When 5 mM of the three BCAAs (leucine, isoleucine, and valine) was added together, both the maximum cell density and growth rate (almost twofold) of MG*bcaP* were increased significantly (Fig. 2A). The addition of 15 mM BCAAs fully restored growth (data not shown), while growth of the wild-type strain remained unchanged. In contrast, growth of MG*bcaP* was not enhanced when the medium was supplemented with the same amount of proline, alanine, and histidine (Fig. 2A) and all 14 other non-branched-chain amino acids (data not shown).

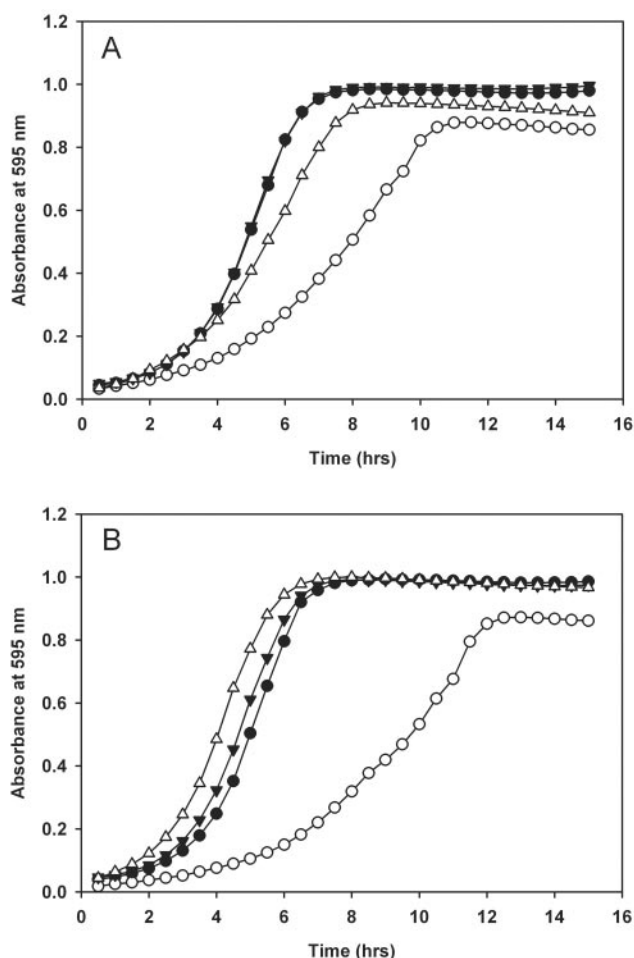

FIG. 2. Growth of *L. lactis* MG1363 and MG*bcaP* in the presence of different amino acids and dipeptides. (A) Growth of *L. lactis* MG1363 and MG*bcaP* in CDM supplemented with amino acids P/A/H (closed and open circles, respectively) or I/L/V (closed and open triangles, respectively). (B) Growth of *L. lactis* MG1363 and MG*bcaP* in CDM supplemented with dipeptides PG/AH (closed and open circles, respectively) or LI/PV (closed and open triangles, respectively). Cells were grown at 30°C, and the optical density of the culture at 595 nm was measured every 30 min. Shown are the means of at least two independent experiments.

In accordance with these data, the addition of a mixture of BCAA-containing dipeptides (i.e., LI and PV) fully restored the growth of MG*bcaP*, whereas such a stimulatory effect was

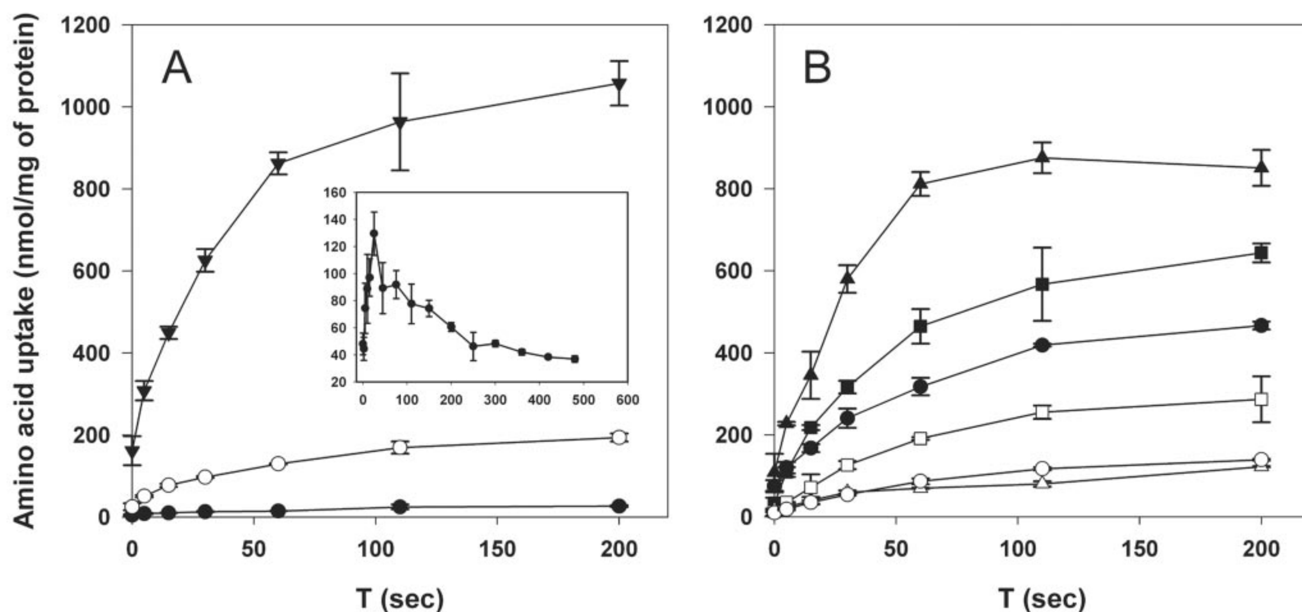

FIG. 3. Time course of uptake of BCAAs and methionine in whole cells of *L. lactis*. (A) Uptake of L-isoleucine by *L. lactis* MG1363 (open circles), MG*bcaP* (closed circles), and MG*bcaP* (pNG*bcaP*) overproducing BcaP (closed triangles). L-[^{14}C]leucine uptake in the presence of 100 μM of unlabeled substrate and in the absence of glucose in the uptake buffer is shown in the inset. (B) Uptake by *L. lactis* MG1363 and *L. lactis* MG*bcaP* (pNG*bcaP*) of L-leucine (open and closed triangles, respectively), L-valine (open and closed squares, respectively), and L-methionine (open and closed circles, respectively). Transport assays using concentrated samples of cells harvested from the exponential phase of growth were performed in the presence of 0.05 μCi ^{14}C- or ^{35}S-labeled amino acid and 250 μM of the corresponding unlabeled amino acid as described in Materials and Methods. Prior to the uptake assay, cells were energized by incubation for 5 min at 30°C in the presence of 0.5% glucose. T, time.

absent when dipeptides lacking BCAA residues were provided (Fig. 2B). The growth defect of MG*bcaP* was already bypassed at a concentration of 500 μM of the dipeptides, most probably because *L. lactis* has several highly efficient transport systems for dipeptides and contains multiple intracellular peptidases that generate free amino acids from dipeptides (28).

BcaP specifically transports branched-chain amino acids and, to a lesser extent, methionine. Since growth of the *bcaP* mutant could be restored by increasing BCAA concentrations in the medium, the lactococcal *bcaP* gene could encode a BCAA transporter. To test this possibility, the abilities of *L. lactis* MG1363 and MG*bcaP* to transport radioactively labeled L-isoleucine were compared (Fig. 3A). Deletion of *bcaP* resulted in a reduction of the initial rate of isoleucine transport by approximately ninefold, indicating that BcaP is responsible for most of the isoleucine uptake in *L. lactis* under these conditions. Complementation of BcaP function by overproducing BcaP in MG*bcaP* resulted in a strong increase (approximately 70-fold) in the uptake rate of L-[^{14}C]isoleucine. Increasing the ratio of L-[^{14}C]isoleucine over unlabeled isoleucine by lowering the concentration of unlabeled isoleucine in the reaction mixture from 250 to 100 μM or omitting glucose from the uptake buffer resulted in a more rapid internalization of L-[^{14}C]isoleucine followed by a slower efflux of the isotope (Fig. 3A). Such kinetics are indicative of so-called counterflow transport and suggest that BcaP is able to facilitate bidirectional transport and most likely constitutes a secondary transporter (14, 63).

To estimate the substrate specificity of BcaP, inhibition of isoleucine transport by other amino acids was monitored in a competition experiment (Fig. 4). The addition of a 10-fold excess of isoleucine or leucine resulted in a reduction of uptake of about 80%, whereas valine was less inhibitory (64%). In addition to the BCAAs, transport of isoleucine by BcaP was inhibited by the sulfur-containing amino acids methionine

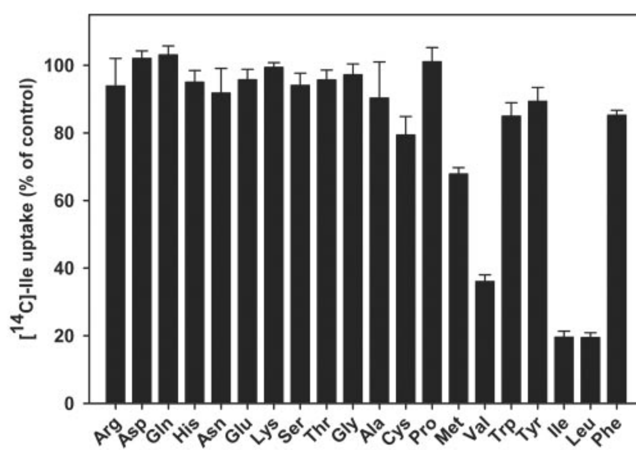

FIG. 4. Substrate specificity of *L. lactis* MG1363 BcaP as determined by the inhibition of L-[^{14}C]isoleucine uptake by L-amino acids. Shown is the percentage of L-[^{14}C]isoleucine uptake by lactococcal cells overproducing BcaP in the presence of 250 μM unlabeled L-isoleucine (control) and a 10-fold excess of each of the 20 L-amino acids. The uptake was determined after 1 min of incubation at 30°C, and the assay was performed as described in the legend to Fig. 3. The means of three independent measurements are shown.

TABLE 3. Homologs of *L. lactis* MG1363 BcaP in several species of gram-positive bacteria

Species	Protein[a]	Protein length (amino acids)	Identity (%)	Similarity (%)	Reference or source
Lactococcus lactis MG1363	BcaP (CtrA)	465	100	100	This work
Lactococcus lactis IL1403	CtrA	469	98	99	4
Staphylococcus aureus COL	YP_187408	482	64	80	15
Lactobacillus casei ATCC 334	ZP_00384379	464	40	61	[b]
Lactobacillus plantarum WCFS1	Lp_0861	465	39	61	27
Listeria innocua	Lin0648	463	36	61	16
Enterococcus faecalis V583	NP_814667	463	34	58	47
Bacillus cereus G9241	ZP_00238192	471	37	57	21
Bacillus subtilis 168	YhdG	465	33	56	34
Streptococcus pneumoniae R6	YfnA	467	34	54	23
Streptococcus thermophilus CNRZ1066	Str1361	461	33	53	3

[a] In case no protein name was assigned, the locus tag of the encoding ORF is shown.
[b] Part of an ongoing genome-sequencing project, the sequence data of which are available online (http://www.doe.jgi.gov).

(23% inhibition) and, to a lesser extent, cysteine. These results indicate that the ability of BcaP to bind amino acids increases as a function of their hydrophobicity, with the exception of those amino acids containing bulky side chains (i.e., Trp, Tyr, and Phe).

To test whether BcaP is capable of transporting the amino acids it recognizes, time course experiments were performed as described above for isoleucine by using radioactively labeled leucine, valine, and methionine. Consistent with the competition experiments, the transport rate of BcaP for leucine was comparable to that for isoleucine, and the rate for valine was higher than that for methionine (Fig. 3B). Uptake rates for these substrates by the *bcaP* mutant strain were similar to that of isoleucine (data not shown). No significant difference in transport of the nonbinding amino acid proline between the *bcaP* mutant and the BcaP-overproducing strain was observed (data not shown).

BcaP is conserved in several gram-positive bacteria. The transport and growth experiments clearly show that BcaP specifically transports branched-chain amino acids and methionine, which is in disagreement with its predicted function. A BLAST search (1) with the full-length amino acid sequence against the NCBI nonredundant protein database revealed that homologs of BcaP are contained in the genomes of several gram-positive bacteria (Table 3). The amino acid sequence of lactococcal BcaP is similar to that of YhdG of *B. subtilis*. Like *bcaP*, transcription of *yhdG* has been shown to be strongly CodY dependent (44). Therefore, it is likely that *L. lactis* BcaP and *B. subtilis* YhdG represent functional homologs. Homologs of BcaP are present in species of *Listeria*, *Streptococcus*, *Staphylococcus*, and *Lactobacillus*, which all contain (putative) homologs of CodY (56). Notably, in the latter bacterium, a second homolog that is even more similar to lactococcal BcaP was found, although it has a C-terminal extension of about 34 amino acid residues that is not present in the *L. lactis* protein.

The amino acid sequences of the BcaP homologs are extensively conserved throughout the proteins, with the exception of the N-terminal parts. A hydropathy analysis of BcaP, using the method described previously by Kyte and Doolittle (35), predicts 12 transmembrane segments (data not shown). These transmembrane helices, with an average size of 23 amino acid residues, are linked by short hydrophilic stretches that vary in size from 3 to 40 residues. Both the N and C termini are predicted to protrude into the cytoplasm. The hydrophobicity profile of BcaP is conserved in all homologs. Among the 465 amino acid residues constituting BcaP, 326 are hydrophobic (70%), 87 are polar (18.7%), 18 are acidic (3.9%), and 34 are basic (7.3%) amino acid residues, respectively. Thus, BcaP is strongly hydrophobic, which is a typical feature of integral membrane transporters.

Role of BcaP in global gene expression in *L. lactis*. As transcription of *bcaP* is tightly regulated by the global regulator CodY and the absence of *bcaP* is deleterious for the cells under amino acid-limiting conditions (Fig. 1), the effect of the removal of *bcaP* on global gene expression was monitored by comparing the transcriptomes of *L. lactis* MG1363 and *L. lactis* MG*bcaP*. Both strains were grown in CDM containing free amino acids as the sole amino acid source. RNA samples were prepared from each strain and, following cDNA synthesis and labeling, hybridized to DNA microarrays representing 2,450 genes of *L. lactis* MG1363. Analysis of the DNA microarray data of four biological replicates revealed that the expression of approximately 20 genes or operons was significantly up-regulated, while the expression of approximately 20 genes or operons was down-regulated upon mutation of *bcaP* (Table 4). The majority of the up-regulated transcriptional units are involved in (branched-chain) amino acid metabolism or peptide transport. Some of these (e.g., *dpp*, *ilv*, and *his*) belong to the regulon of the transcriptional regulator CodY. The extent of derepression seemed less than that observed in a strain deleted for *codY* (6), which might be explained, because CodY is still able to regulate its targets at low levels of BCAAs or in the absence of these cofactors (5). Interestingly, expression of *cysD*, *cysK*, *cysM*, and *metB2* was significantly increased in MG*bcaP*. Expression of these genes, encoding enzymes required for methionine and cysteine biosynthesis, has recently been shown to be controlled by the transcriptional activator CmbR (57). Thus, BcaP provides a link between sulfur amino acid biosynthesis and metabolism of BCAAs in *L. lactis*.

BrnQ contributes to BCAA transport in *L. lactis*. Although BcaP is responsible for most of the BCAA uptake in *L. lactis* (Fig. 3), MG*bcaP* can still grow in CDM containing only free amino acids (Fig. 1). Since *L. lactis* MG1363 is auxotrophic for BCAAs, the strain must contain at least one other transport system for BCAAs that is active under those conditions. *L. lactis* MG1363 contains a homolog of BrnQ, a low-affinity

TABLE 4. Comparison of the transcriptomes of *L. lactis* MG*bcaP* and *L. lactis* MG1363

Transcriptional unit[a]	Expression ratio[b]	Significance (*P* value)	Description[c]	Regulon
dppA, *P*, *B*, *C*, *D*, *F*	4.5	10^{-8}	Dipeptide transport system	CodY
ybbE	3.0	10^{-13}	Hypothetical protein, downstream of *bcaP*	
llmg_0328	2.4	10^{-6}	Hypothetical protein	
comX	2.2	10^{-9}	Putative regulator	
gltA, *citB*, *icD*	2.1	10^{-13}	Krebs TCA cycle enzymes	CodY
leuC, *A*	2.0	10^{-9}	Leucine biosynthesis	CodY
hisC, *Z*, *G*, *D*, *B*, *ymdC*, *hisH*, *A*, *F*, *I*, *K*	1.9	10^{-10}	Histidine biosynthesis	CodY
metC-cysK	1.9	10^{-8}	Methionine/cysteine synthesis	CmbR
rmaG	1.9	10^{-4}	Transcriptional regulator	
llmg_1066	1.8	10^{-6}	Putative membrane protein	
llmg_0472/0473	1.7	10^{-5}	Hypothetical proteins	
ilvD, *B*, *N*, *C*, *A*, *aldB*	1.6	10^{-7}	BCAA biosynthesis	CodY
fabI	1.6	10^{-5}	Enoyl-ACP reductase	
purC, *H*	1.6	10^{-7}	Purine synthesis	CmbR
cysD	1.6	10^{-11}	*O*-Acetylserine sulfhydrylase	CmbR
ydbE	1.6	10^{-4}	Hypothetical protein	
glgD	1.6	10^{-6}	Glucose-1-phosphate adenylyltransferase	CmbR?
yrbB	1.5	10^{-10}	Hypothetical protein	
yriD	1.5	10^{-6}	Hypothetical protein	CmbR
cysM	1.5	10^{-7}	Cysteine synthase	CmbR
cpo	0.7	10^{-6}	Hydrolase or acyltransferase	
yrcA	0.7	10^{-7}	Phospho-beta-glucosidase	
yjaE	0.7	10^{-4}	Hypothetical protein	
ynjC	0.7	10^{-4}	Hypothetical protein	
argF	0.7	10^{-6}	Ornithine carbamoyltransferase	
yedF	0.6	10^{-7}	PTS II ABC component	
trpB	0.6	10^{-6}	Tryptophan synthase beta chain	
butB	0.6	10^{-6}	Dehydrogenase	
ptcA	0.6	10^{-5}	Cellobiose-PTS component	
panE	0.6	10^{-9}	Ketopantoate reductase	
fruA	0.6	10^{-5}	Fructose-PTS component	
pgmB	0.5	10^{-9}	Beta-phosphoglucomutase	
busAB	0.5	10^{-8}	Betaine ABC transporter	
cstA	0.5	10^{-4}	Carbon starvation protein	
bglS, *ybhD*	0.5	10^{-6}	Beta-glucosidase A, hypothetical protein	
yecA	0.5	10^{-5}	Transcriptional regulator	
chiA, *yucG*	0.4	10^{-5}	Chitinase, chitin-binding protein	
rcfB, *yxbD*	0.4	10^{-4}	Transcriptional regulator, transporter	
argH	0.4	10^{-7}	Argininosuccinate lyase	
yjjA	0.3	10^{-14}	Hypothetical protein	

[a] The gene (in an operon) that shows the highest ratio of expression is underlined.
[b] Ratio of expression in *L. lactis* MG*bcaP* over that in *L. lactis* MG1363.
[c] (Putative) gene function. TCA, tricarboxylic acid; ACP, acyl carrier protein; PTS, phosphotransferase system.

BCAA carrier in a number of gram-positive bacteria including *Lactobacillus delbruckii* (59). To investigate whether BrnQ might serve a similar purpose in *L. lactis* MG1363, the gene was deleted from the chromosome, and the growth characteristics of the resulting strain (MG*brnQ*) were examined (Fig. 5). Unlike for MG*bcaP*, no significant growth difference between *L. lactis* MG1363 and MG*brnQ* grown in CDM[75] was observed. In contrast, a strain in which both *brnQ* and *bcaP* were deleted (MG*bcaPbrnQ*) was not viable in this medium. After a prolonged lag phase, the double-mutant strain did grow in standard CDM, but growth was significantly hampered (ninefold), and the culture reached a lower final cell density than the wild-type strain (1.5-fold). Growth of the double mutant was enhanced by the addition of BCAAs to the CDM. No differences in growth behavior between wild-type, mutant, and double-mutant strains were observed in ½GM17 or in CDM supplemented with Casitone (data not shown). These results indicate that *brnQ* most likely serves as a second, but less efficient, BCAA transport system in *L. lactis* MG1363.

DISCUSSION

During the past decades, various studies have led to the identification of transport systems responsible for the uptake of amino acids in LAB (29). Although many of the properties of these carriers were revealed, most of the genetic elements involved remained elusive. In the current study, a tightly regulated and conserved gene encoding a putative cationic amino transporter, CtrA (BcaP), was cloned, and its protein product was functionally characterized. For several reasons we conclude that the encoded protein indeed constitutes an amino acid transporter that, in contrast to its predicted function, specifically transports BCAAs and, to a lesser extent, methionine. Firstly, the growth defect brought about by the removal

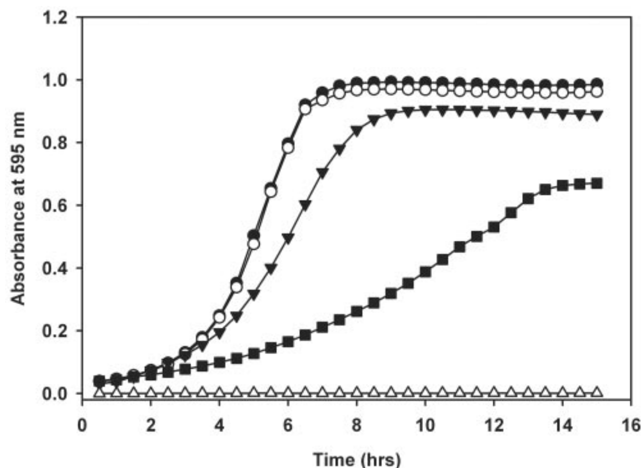

FIG. 5. Growth of *L. lactis* MG1363, MG*brnQ*, and MG*bcaPbrnQ* in different media. Growth levels of *L. lactis* MG1363 (closed circles), MG*brnQ* (open circles), and MG*bcaPbrnQ* (open triangles) in CDM^{75}, MG*bcaPbrnQ* (closed triangles) in CDM^{75} supplemented with amino acids I/L/V, and MG*bcaPbrnQ* (squares) in standard CDM are shown. Cells were grown at 30°C, and the optical density of the culture at 595 nm was measured every 30 min. Shown are the means of at least two independent experiments.

of *bcaP* from the chromosome was complemented by the addition of BCAAs or BCAA-containing dipeptides to the medium and not by those containing other amino acids. Secondly, the *bcaP* deletion strain showed a strongly reduced uptake activity of BCAAs and methionine compared to that of its parental strain. Finally, overexpression of plasmid-encoded CtrA in MG*bcaP* resulted in a significant increase in the uptake of BCAA and methionine. In view of its newly identified function, we have renamed CtrA BcaP, for branched-chain amino acid permease.

We show that the presence of *bcaP* is beneficial to *L. lactis* when it is grown in CDM containing free amino acids as the sole amino acid source and that this advantage is lost when a complex mixture of peptides (in the form of Casitone) is added to the medium (Fig. 1). When peptides are available, BcaP is probably superfluous, as intracellular BCAA pools can be maintained through the uptake and intracellular cleavage of peptides into free amino acids. Complementation of growth of the *bcaP* mutant was already achieved upon the addition of 500 μM of BCAA-containing dipeptides, which are substrates for the highly efficient transport systems encoded by *dpp* (52, 53) and *dtpT* (12, 20), whereas concentrations of the corresponding free amino acids in CDM need to be over 10-fold higher for full complementation. It remains to be established which physiological role BcaP serves for *L. lactis* growing in milk, as amino acid concentrations in this environment are low and oligopeptides are the main source of nitrogen (25). It may be that BcaP facilitates the excretion of redundant BCAAs under these conditions.

The absence of an apparent physiological function of BcaP during growth under peptide-rich conditions could explain why its transcription is tightly regulated by CodY, the master regulator of the genes constituting the proteolytic system of *L. lactis* (6). The *bcaP* gene is one of the main targets of the repressor, and it has been shown that *bcaP* transcription is directly regulated by CodY (6). CodY regulates its targets through a so-called CodY box, a DNA motif that serves as a high-affinity binding site for CodY. Upstream of *bcaP*, no less than three copies of the CodY box are present, which might reflect the stringent regulation of *bcaP* by CodY. As CodY activity itself is modulated by BCAAs (5, 48, 54), the regulator is expected to be fully active when BCAAs or peptides containing BCAAs are abundant. However, it appears that *bcaP* is not fully repressed in peptide-rich medium, as a difference in isoleucine uptake between the isogenic wild-type and *bcaP* mutant strains could still be discerned when both were grown in GM17 (Fig. 3). Preliminary experiments indicate that in a strain in which *codY* is deleted, some peptide-dependent regulation of *bcaP* transcription still occurs (data not shown). This suggests the presence of a second, CodY-independent mechanism of repression of *bcaP* transcription.

As for many LAB, *L. lactis* MG1363 is auxotrophic for a number of amino acids (BCAAs, Met, and His). Since cells of MG*bcaP* are viable in media containing free amino acids as the only amino acid sources, an additional transport system that retrieves BCAAs from the growth medium must be active under these conditions. A homolog of the well-conserved BCAA permease BrnQ (45, 59, 60) is specified by the chromosome of *L. lactis* MG1363, and our data suggest that the product of this gene is responsible for part of the BCAA uptake in MG*bcaP*. A region of BcaP is homologous to a stretch of amino acids of BrnQ, which might be an indication that this region contains a substrate (BCAA) recognition domain. Future studies will be required to detail structure-function relationships of these conserved transport systems.

Previously, a lactococcal amino acid carrier with specificity for BCAAs was characterized biochemically using membrane isolates, but no gene encoding this carrier was identified (8–10). Transport of BCAAs by the carrier was inhibited by L-amino acids containing branched or aliphatic side chains with at least three methyl groups. As amino acid uptake by BcaP is strongly inhibited by BCAAs and methionine, the latter of which can be regarded as an aliphatic amino acid since the S atom in the side chain is largely nonreactive, the data presented in the current study indicate that *bcaP* might be the structural gene encoding this unknown carrier described previously by Driessen and coworkers (8–10). Moreover, those authors have shown that substrates of the transport system are translocated in symport with one proton and that transport can be bidirectional, which fits well with our observations that BcaP constitutes a secondary transport system that enables the counterflow of its substrates. However, part of the BCAA uptake by *L. lactis*, as observed previously by Driessen and coworkers, might be due to BrnQ activity. BrnQ was characterized as a low-affinity BCAA carrier in *Lactobacillus delbruckii* (59). In *L. lactis*, BrnQ activity is probably also responsible for only a minor fraction of total BCAA uptake, at least under the conditions examined in this work, as the functional removal of *bcaP* resulted in a loss of most of the isoleucine transport capacity (Fig. 3) and deletion of *brnQ* alone did not have any effect on growth (Fig. 5). The redundancy of BCAA transport systems in *L. lactis* stresses the importance of BCAAs for the cell. BCAAs serve as precursors for branched-chain fatty acids and are the most abundant amino acids in membrane proteins. Transcription of *L. lactis brnQ* was recently

shown to be affected by environmental stress (65). In *Salmonella enterica* serovar Typhimurium and *Corynebacterium glutamicum*, transcription of *brnQ* is modulated by BCAAs (37, 46), but the mechanisms remain unknown. Future work will have to show whether expression of *brnQ* in *L. lactis* is regulated.

Surprisingly, the strain in which both *brnQ* and *bcaP* were deleted was still viable in CDM containing only free amino acids, although growth started only after a prolonged lag phase and the culture reached a much lower final cell density (Fig. 5) than the wild-type or single-mutant strains. These results suggest the presence of at least one more system that enables BCAA transport in *L. lactis*. Alternatively, the cells might acquire the necessary BCAAs through passive diffusion via the membrane. To identify candidates that could be responsible for the remaining BCAA influx, we searched the lactococcal genome for other putative BCAA uptake system genes. The product of *ydgC* shares homology with Aap, a general amino acid permease of *Rhizobium leguminosarum* (64). Transcription of this gene, like *bcaP*, is dependent on CodY (6) and might therefore be important for BCAA metabolism. In addition, *L. lactis* MG1363 contains a copy of *azl*, an operon suggested to be involved in BCAA transport in *B. subtilis* (2). Future work will aim to establish whether lactococcal *ydcG* and *azl* encode functional proteins and whether they are involved in BCAA transport.

A number of genes encoding enzymes involved in sulfur amino acid metabolism were differentially expressed upon removal of *bcaP* (Table 4). In *L. lactis*, most of these genes are controlled by CmbR (11, 57). The activity of this LysR-type transcriptional activator is modulated by *O*-acetyl serine (17), a precursor in cysteine biosynthesis. In addition, it has been suggested that other regulatory mechanisms might be important for coordinating sulfur metabolism (57). It could be that the changes in the expression of sulfur metabolism genes, as a consequence of the *bcaP* mutation, are the result of changes in the intracellular pool of methionine. Similarly, up-regulation of many target genes of the pleiotropic regulator CodY in MG*bcaP* might be explained by lowered intracellular BCAA levels in this strain, leading to the inactivation of CodY and the concomitant derepression of genes belonging to the CodY regulon. Most of these genes encode transporters and amino acid biosynthetic enzymes that could help to counteract the effects of the deletion of *bcaP*. Since BcaP is responsible for most of the uptake of BCAAs and (at least) part of the methionine, this transporter provides a link between BCAA and sulfur amino acid metabolism.

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