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The *bspA* Locus of *Lactobacillus fermentum* BR11 Encodes an L-Cystine Uptake System

MARK S. TURNER,[1] TONIA WOODBERRY,[2] LOUISE M. HAFNER,[1] AND PHILIP M. GIFFARD[1]*

Centre for Molecular Biotechnology, School of Life Sciences, Queensland University of Technology,[1] and Queensland Institute of Medical Research, Herston,[2] Brisbane, Australia

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BspA is a basic surface-exposed protein from *Lactobacillus fermentum* BR11. Sequence comparisons have shown that it is a member of family III of the solute binding proteins. It is 89% identical to the collagen binding protein, Cnb, from *Lactobacillus reuteri*. Compared with the database of *Escherichia coli* proteins, BspA is most similar to the L-cystine binding protein FliY. To investigate the function of BspA, mutants depleted for BspA were generated by homologous recombination with a temperature-sensitive plasmid. These mutants were significantly impaired in their abilities to take up L-cystine. Uptake rates of L-glutamine, L-histidine, and L-lysine, which are substrates for other binding proteins with similarity to BspA, were unaffected. Evidence was obtained that BspA is necessary for maximal resistance to oxidative stress. Specifically, inactivation of BspA causes defective growth in the presence of oxygen and sensitivity to paraquat. Measurements of sulfhydryl levels showed that incubation of *L. fermentum* BR11 with L-cystine resulted in increased levels of sulfhydryl groups both inside and outside the cell; however, this was not the case with a BspA mutant. The role of BspA as an extracellular matrix protein adhesin was also addressed. *L. fermentum* BR11 does not bind to immobilized type I collagen or laminin above background levels but does bind immobilized fibronectin. Inactivation of BspA did not significantly affect fibronectin binding; therefore, we have not found evidence to support the notion that BspA is an extracellular matrix protein binding adhesin. As BspA is most probably not a lipoprotein, this report provides evidence that gram-positive bacterial solute binding proteins do not necessarily have to be anchored to the cytoplasmic membrane to function in solute uptake.

Binding protein-dependent ATP-binding cassette (ABC)-type systems have been found to mediate the uptake of solutes such as amino acids, peptides, ions, vitamins, and sugars in a wide range of bacteria (15, 33). Components of these systems include one or two ATP-binding proteins localized to the cytoplasmic side of the cytoplasmic membrane, one or two hydrophobic transmembrane proteins forming the solute-specific channel, and a high-affinity solute binding protein external to the cytoplasmic membrane (33). In gram-negative bacteria the solute binding protein diffuses freely within the periplasm, while in gram-positive bacteria the solute binding protein is normally covalently anchored to a lipid molecule in the cytoplasmic membrane. This is thought to prevent its loss to the environment (32).

We have recently characterized the basic surface-exposed protein, BspA, from *Lactobacillus fermentum* BR11 (35). This protein can be selectively removed from whole cells by using 5 M LiCl or a low-pH buffer, indicating that it is likely to be anchored noncovalently to the cell surface (35). Sequence alignments have shown that BspA is a member of family III of the bacterial solute binding proteins (33), and open reading frames upstream of *bspA* potentially encode the other components of an ABC-type uptake system (35). Members of the family III solute binding proteins have been shown to bind polar amino acids and opines such as cystine, glutamine, arginine, histidine, lysine, octopine, and nopaline (33). BspA is unusual in that it does not possess the lipoprotein consensus sequence LXXC that has been found in all solute binding proteins in gram-positive bacteria so far identified. This is, however, consistent with its apparent electrostatic anchoring mechanism. A comparison of BspA to all the proteins encoded by the *Escherichia coli* genome revealed that BspA is most similar to the L-cystine binding protein FliY. Here we report that the *L. fermentum* BR11 *bspA* locus, and specifically BspA, is required for L-cystine uptake. We also report that this L-cystine uptake system is required for the production of intracellular and extracellular sulfhydryl compounds from L-cystine and for maximal resistance of *L. fermentum* BR11 to the superoxide radical-generating chemical, paraquat.

Recently, two members of the family III solute binding proteins have been characterized on the basis of adhesin function rather than involvement in solute uptake. These are the collagen binding protein Cnb from *Lactobacillus reuteri* (28) and the PEB1 adhesin from *Campylobacter jejuni* (25). BspA is 88.6% identical to Cnb, so its contribution to cell adhesion was addressed. Here we show that BspA does not appear to confer upon whole *L. fermentum* BR11 cells the ability to bind immobilized type I collagen, laminin, or fibronectin.

MATERIALS AND METHODS

Strains and plasmids. *L. fermentum* BR11 is a guinea pig vaginal tract isolate that has previously been described (29). It was grown in liquid or on solid MRS medium (Oxoid) anaerobically or in the presence of 5% CO_2 at indicated temperatures. *E. coli* JM109 (39) and recombinant derivatives used in molecular cloning experiments were grown in Luria-Bertani medium (30) at 37°C, with shaking. The *E. coli-Streptococcus* shuttle plasmid pJRS233 has previously been described (26). As well as the temperature-sensitive broad host range replicon from pG+host4, pJRS233 contains the low-copy-number replicon pSC101, thus allowing stable propagation in *E. coli* at 37°C. Erythromycin was used at concentrations of 750 μg/ml for *E. coli* and 10 μg/ml for *L. fermentum* BR11, PNG201, and PNG202, unless otherwise stated.

Disruption of the *bspA* locus to construct PNG201. In order to disrupt the *bspA* locus and abolish expression of BspA, we devised a strategy to separate the *bspA* gene from its promoter by integrating a plasmid just upstream of *bspA* via homologous recombination. The homologous DNA that was used upstream from

* Corresponding author. Mailing address: Centre for Molecular Biotechnology, School of Life Sciences, Queensland University of Technology, G.P.O. Box 2434, Brisbane, Queensland 4001, Australia. Phone: (61-7) 3864-2015. Fax: (61-7) 3864-1534. E-mail: p.giffard@qut.edu.au.

bspA was amplified from plasmid pMFT3 (35) with primers 5′-AATTATCTAG ACATCCTTACCACAACT-3′ and 5′-CATCGAAGCTTTAAAGTTTTTAAT CCG-3′, and the 1.4-kb product was purified from an agarose gel with Bresaclean (Bresatec), cleaved with *Xba*I-*Hin*dIII, and ligated to *Xba*I-*Hin*dIII-cleaved pJRS233 to yield pPNG201. Plasmid pPNG201 was propagated in *E. coli* prior to electroporation into *L. fermentum* BR11 (see below). It was predicted that while disruption of the *bspA* locus would not alter the *orf2* product (putative hydrophobic membrane protein), it would modify the product of *orf3* (putative ATP-binding protein) (35). The last 10 amino acids of the *orf3* product would be changed, and another 13 amino acids would be added onto the carboxyl terminus of this protein according to the sequence of the pJRS233 plasmid. To confirm that disruption of the *bspA* locus had occurred, Southern hybridization was performed with a digoxygenin-11-dUTP PCR-labelled probe according to the manufacturer's instructions (Boehringer Mannheim) and detected by using disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) (Boehringer Mannheim). The 600-bp probe was amplified from pMFT3 (35) with primers 5′-TATGGTGATGCCTATCC-3′ and 5′-TCAAAACCAGTCAGCTTG-3′. *Hin*dIII-cleaved λ DNA, labelled with digoxigenin (DIG)-dUTP with Ready-to-go DNA labelling beads (Pharmacia), was also used as a probe in Southern hybridization. Chromosomal DNA for the Southern hybridization was isolated from *L. fermentum* BR11 and PNG201 by the method previously described (35). PCRs used for cloning DNA into plasmids were performed by using the Expand High Fidelity PCR System (Boehringer Mannheim) according to the manufacturer's instructions.

Construction of PNG202. During our current research into the potential utility of BspA as a cell surface presentation vector, a chimeric gene was constructed consisting of *bspA* fused at its 3′ end to the DNA sequence GGTTCAGGAA TTCGTATCCTGGCCGTCGAACGTTATCTGAAAGATCAGCAGCTGCT GGGCATCTGGGGCTGCTCTGGCAAACTGATCTGCACCACGGCGGT CCCGTGGAATGCGTCTTAA. This sequence encodes a hydrophilic peptide linker and a fragment of the human immunodeficiency virus (HIV) gp41 protein. The chimeric gene was inserted upstream of the putative *bspA* terminator and then cloned into pJRS233, which was integrated into the *L. fermentum* BR11 genome similarly as for pPNG201. Sequence analysis of the chimeric gene revealed that a C-to-T misincorporation at nucleotide 346 of *bspA* resulting in a stop codon had occurred during PCR amplification. It was therefore predicted that this integrant (PNG202) would be specifically deficient in BspA expression.

Transformation of *L. fermentum* BR11. Transformation of *L. fermentum* BR11 with plasmids was done by using a procedure similar to that described by Wei et al. (37). A stationary-phase *L. fermentum* BR11 culture was diluted 66-fold in MRS and then grown for 3 h. Penicillin then was added to a final concentration of 1.25 μg/ml, and the cells were further incubated for 1.5 h. The cells were harvested, washed twice in cold 5 mM sodium phosphate–1 mM $MgCl_2$ (pH 7), and resuspended in 1% of the original culture volume in cold electroporation buffer (0.9 M sucrose, 3 mM $MgCl_2$ [pH 7.4]). Plasmid DNA (100 ng to 1 μg) was mixed with 100 μl of cells in a 0.2-cm-diameter cuvette (Bio-Rad). Electroporation conditions were as follows: peak voltage, 2.5 kV; capacitance, 25 μF; and parallel resistance, 200 Ω. Subsequent to pulsing, cells were incubated in 1 ml of prewarmed MRS medium for 1 h at 30°C, after which time erythromycin resistance was induced by the addition of erythromycin to 0.01 μg/ml and continued incubation at the same temperature for approximately 20 h. Cells then were plated onto MRS agar containing 10 μg of erythromycin/ml and incubated at 30°C.

Cell surface extractions and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. LiCl extractions of *L. fermentum* BR11 and PNG201 were done as described previously (35), except that the cells were kept at 24°C during the LiCl extractions rather than at 0°C. This resulted in an increase in the quantity of extracted BspA and the extraction of another protein from the cells that was used as an extraction efficiency control during the screening of putative *bspA* integrants.

Amino acid uptake assays. Cells grown to mid-exponential phase were harvested by centrifugation, washed twice in KPM solution (0.1 M K_2HPO_4 adjusted to pH 6.5 with H_3PO_4 and containing 10 mM $MgSO_4 \cdot 7H_2O$) (17), and suspended in KPM to a density of 3.9×10^8 cells per ml. Portions of this cell suspension (0.5 ml each) were energized, when appropriate, by the addition of 9.5 μl of 1 M D-glucose, followed by incubation for 7 min at 37°C. Uptake was initiated by the addition of 0.12 μCi of L-[^{14}C]cystine (76 mCi/mmol) (NEN) or 0.48 μCi of L-[^{14}C]glutamine (277 mCi/mmol), L-[^{14}C]histidine (303 mCi/mmol), or L-[^{14}C]lysine (316 mCi/mmol) (Amersham). Samples were removed at intervals, cells were immediately collected onto washed GF/F filters (Whatman), and their radioactivity was determined by liquid scintillation.

Determination of intracellular and extracellular sulfhydryl levels. This method is based on that of Thomas (34) and involved incubation of cells with L-cystine and glucose and then measurement of the sulfhydryl compounds intracellularly and extracellularly with the chromogenic disulfide compound 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma). Cells were grown and washed as for the amino acid uptake assays and then suspended in KPM to a density of 3.6×10^8 cells per ml. Portions (0.5 ml each) of this suspension were supplemented with 10 μl of 10 mM L-cystine and 10 μl of 1 M D-glucose, and the suspension was then incubated at 37°C for 1 h. The cells were then pelleted, and the supernatant was removed and put on ice. Fifty microliters of a 10 mM solution of DTNB in KPM was added to the supernatant, and the absorbance at 412 nm was measured. The pelleted cells were resuspended, washed twice with 1 ml of KPM, and then resuspended in a solution containing 200 μl of water, 4 μl of 0.5 M EDTA, 10 μl of 1 M Tris-HCl (pH 8), 20 μl of 10 mM DTNB, and 100 μl of 10% SDS, added successively. This mixture was incubated at 37°C for 1 h and then centrifuged, and the absorbance of the supernatant at 412 nm was measured. Control assays with either no cells or no L-cystine were also carried out. For determining the sulfhydryl concentrations, the amount of the reduced product (5-thio-2-nitrobenzoic acid) was determined from the absorbance at 412 nm by assuming a molar extinction coefficient of 13,600 (8).

Determination of the effect of paraquat on growth rates. Five hundred microliters of log-phase cells was added to 4.5 ml of prewarmed MRS or MRS and erythromycin containing either 500 μl of sterile double-distilled water (ddH_2O) (0 mM paraquat), 166 μl of 1 M paraquat (methyl viologen; Sigma), and 333 μl of sterile ddH_2O (30 mM paraquat) or 500 μl of 1 M paraquat (90 mM paraquat). The cultures were incubated at 37°C without shaking. Growth was monitored by measuring the optical density at 600 nm (OD_{600}).

Determination of binding of whole cells to immobilized ECM proteins. Microtiter plate wells (Nunc-Maxisorp) were coated with 5 μg of ECM protein in coating buffer (0.02 M Na_2CO_3, $NaHCO_3$ [pH 9.6]) overnight at 4°C. The wells then were emptied, washed three times with phosphate-buffered saline (PBS)-Tween (0.1% Tween 20 [pH 7]), and blocked by treatment overnight with PBS containing 3% skim milk powder at 4°C. The wells were emptied and washed again three times with PBS-Tween, and then aliquots (50 μl) of a suspension (4×10^8 cells per ml) of log-phase PBS (pH 7)-washed cells (equivalent *L. fermentum* BR11 and PNG201 cell numbers were obtained by matching the OD_{600}) were added. The plates were incubated for 1 h at 37°C with rotation (200 rpm), after which the nonadherent cells were removed by washing three times with PBS-Tween. Fifty microliters of an anti-*L. fermentum* BR11 antiserum (35) diluted 1:250 in PBS containing 0.1% bovine serum albumin was then added. The plates were rotated at 24°C for 1 h, and then the wells were washed three times with PBS-Tween. A peroxidase-conjugated anti-rabbit antibody (Dako) diluted 1:1,000 in PBS containing 0.1% bovine serum albumin was added (50 μl), the plates were rotated at 24°C for 1 h, and the wells were washed six times with PBS-Tween. Remaining peroxidase was detected by adding 50 μl of 3,3′,5,5′-tetramethylbenzidine (ELISA Systems, Graphic Scientific Pty. Ltd., Australia) and incubating the plates at 24°C until visible color development had occurred. Then the reaction was stopped by adding 50 μl of 3 M HCl. The color intensity was determined at 450 nm with an automated plate reader.

Chemicals. Type I collagen was obtained from Sigma (catalog no. C-3511). Human fibronectin and mouse laminin were obtained from Boehringer Mannheim.

RESULTS

BspA is more similar to FliY than it is to any other *E. coli* protein. BspA is similar to family III solute binding proteins, which bind a variety of different polar amino acids and opines (33). To provide some insight into which substrate BspA may bind, a BLAST search (2) was performed comparing the sequence of BspA to all of the proteins encoded by the *E. coli* K-12 genome (3). BspA had most homology with FliY (36.2% identity), followed by ArtJ (30.8% identity), GlnH (28.2% identity), ArtI (28.2% identity), ArgT (26.1% identity), HisJ (25.7% identity), and YbeJ (23.4% identity). The percentage identities were calculated by counting each gap as a single mismatch. The significantly higher identity that BspA has with FliY compared to the other proteins indicated to us that these two proteins may function the same way. Purified FliY has been shown to bind L-[^{14}C]cystine (5), and expression of FliY in *E. coli* is induced under sulfate-starvation conditions (27).

Disruption of the *bspA* locus. In order to determine the function of BspA, a strain deficient in BspA expression was constructed as follows. A 1.4-kb region 5′ of the *bspA* gene was cloned into the temperature-sensitive broad host range plasmid pJRS233 to yield pPNG201 (Fig. 1A). pPNG201 was electroporated into *L. fermentum* BR11, and the erythromycin-resistant transformants were grown at the permissive temperature of 30°C. To prevent plasmid replication, the transformants were then incubated in the presence of erythromycin at the nonpermissive temperature of 40°C, thereby allowing only integrants to grow. Approximately one of 1,000 viable cells was found to be erythromycin resistant at the nonpermissive temperature. Southern hybridization with a probe spanning *orf3* and *bspA* (35) confirmed that disruption of the *bspA* locus had

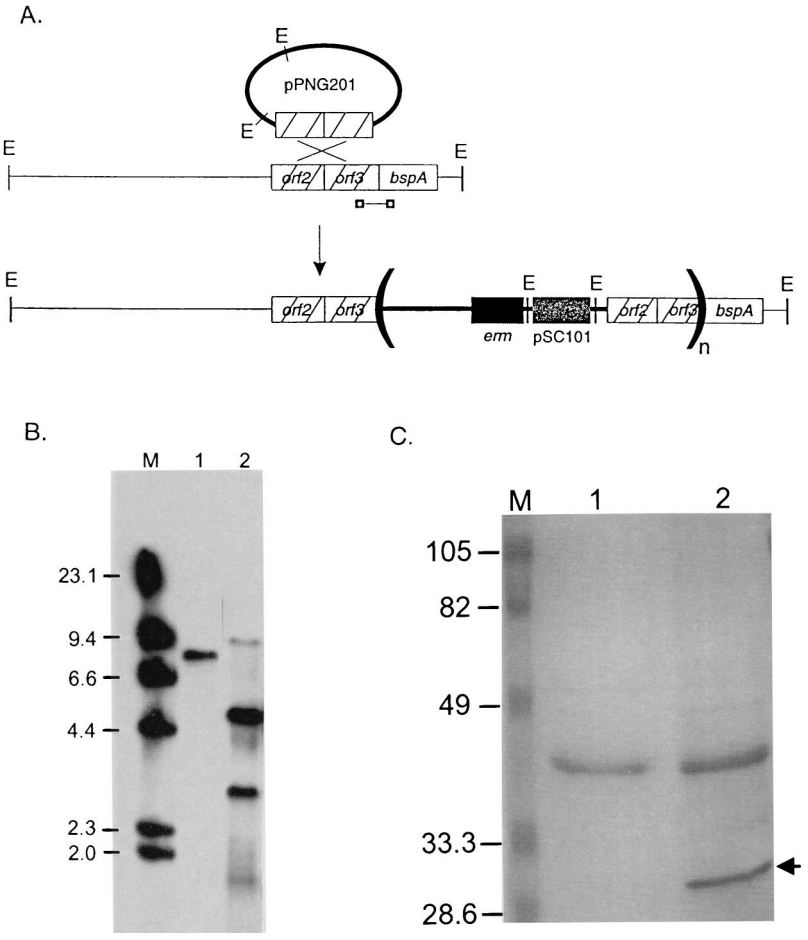

FIG. 1. Disruption of the *bspA* locus. (A) The proposed mechanism for integration of pPNG201 into the *L. fermentum* BR11 chromosome via single crossover homologous recombination. The DNA probe which spans *orf3* and *bspA* is indicated as a dumbbell. The predicted repeat unit is enclosed by brackets. Symbols: hatched box, homologous *L. fermentum* BR11 DNA; E, restriction site for *Eco*RI; black box, erythromycin resistance gene *erm*; grey box, pSC101 plasmid. This diagram is not drawn to scale. (B) Southern hybridization of *L. fermentum* BR11 and PNG201 *Eco*RI-cleaved chromosomal DNA. Lanes: M, *Hin*dIII-cleaved λ DNA (molecular size standards); 1, *L. fermentum* BR11; 2, PNG201. The numbers on the left represent the sizes of the molecular mass markers in kilobases. The origin of the probe is shown in panel A. (C) Coomassie brilliant blue-stained SDS-PAGE analysis of 5 M LiCl extracts from *L. fermentum* BR11 and PNG201. Lanes: M, molecular mass standards; 1, PNG201; 2, *L. fermentum* BR11. The numbers on the left represent kilodaltons. BspA is indicated with an arrow.

occurred (Fig. 1B). The intensity of the 5.3-kb hybridizing band from the integrant PNG201, whose size corresponds to the larger of the two *Eco*RI fragments of pPNG201, indicates that multiple copies of the plasmid were present in the chromosome (Fig. 1B).

To determine whether BspA was expressed by PNG201, a 5 M LiCl extraction of the cells was performed. Coomassie brilliant blue-stained SDS-PAGE of the LiCl extract revealed that BspA was not expressed on the surface of PNG201 (Fig. 1C), thereby confirming that inactivation of *bspA* had occurred. This result demonstrates that pJRS233 can be used as an integration vector in a *Lactobacillus* strain.

The *bspA* locus encodes a high-affinity L-cystine uptake system. To test whether the *bspA* locus encodes proteins involved in L-cystine uptake, the rates of uptake of L-[^{14}C]cystine were compared for *L. fermentum* BR11 and PNG201. It was found that PNG201 was significantly impaired in its ability to take up L-cystine compared to *L. fermentum* BR11. Also, uptake of L-cystine by *L. fermentum* BR11 occurred only when the cells were first energized with glucose (Fig. 2). Interestingly, the intracellular level of ^{14}C in *L. fermentum* BR11 decreased by 42% between the 3- and 5-min time points, suggesting that

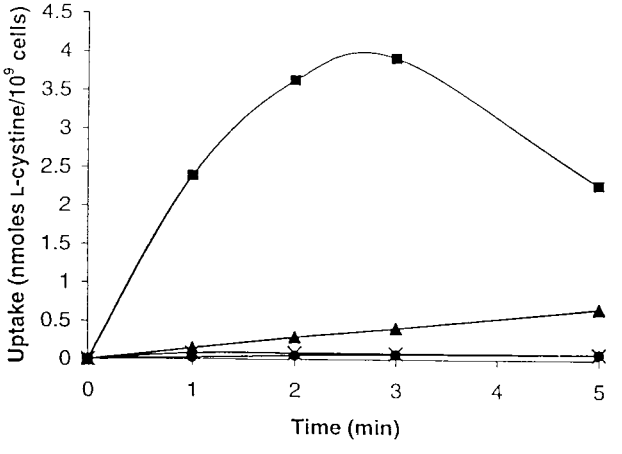

FIG. 2. L-[^{14}C]cystine uptake by *L. fermentum* BR11, PNG201, and PNG202. Uptake in 3 μM L-cystine by *L. fermentum* BR11 (squares), PNG201 (circles), PNG202 (triangles), and *L. fermentum* BR11 which was not energized with glucose (crosses).

either L-cystine or another compound of which L-cystine is a precursor is being exported from the cell. Since the pPNG201 plasmid was integrated upstream of the stop codon of the putative ATP-binding protein-encoding gene (*orf3*), the putative ATP-binding protein from PNG201 has a modified carboxyl terminus and therefore may be nonfunctional in PNG201. Therefore, to confirm that BspA was specifically involved in L-cystine uptake, a mutant (PNG202) which contained a stop codon in its *bspA* gene was also tested for its ability to take up L-cystine. PNG202 contains a *bspA* gene with a TAA stop codon at nucleotides 346 to 348 as well as DNA encoding an epitope from the HIV gp41 protein fused at the 3′ end (see Materials and Methods). PNG202 was found to be significantly impaired in its ability to take up L-cystine compared to the parent strain (Fig. 2); however, it was able to transport more L-cystine than PNG201. Silver-stained SDS-PAGE analysis of a 5 M LiCl extract of PNG202 revealed that a protein the same size as BspA was still being expressed by PNG202 cells, but at greatly reduced levels compared to those expressed by the parent (data not shown). This may be due to read-through of the stop codon in the *bspA* mRNA and the consequent expression of the BspA-gp41 fusion protein, which appears to be functional.

To determine if disruption of the *bspA* locus influenced the uptake rate of some other polar amino acids, the ability of PNG201 to uptake L-[^{14}C]glutamine, L-[^{14}C]histidine, and L-[^{14}C]lysine was compared with that of the parent strain. All were taken up rapidly, and in no case was there a significant difference in uptake rate between the mutant and the parent (data not shown).

PNG201 is more sensitive to oxidative stress than its parent. It was observed during routine culturing that when log-phase PNG201 and *L. fermentum* BR11 cells were plated onto solid media, PNG201 grew significantly slower in the presence of oxygen. This growth rate difference was not observed in the absence of oxygen (data not shown). It was therefore hypothesized that the L-cystine transport-deficient mutant, PNG201, may be more sensitive to oxidative stress because it is unable to take up L-cystine and then convert it to free sulfhydryl compounds, which may protect the cell against oxidative stress. To determine if PNG201 was more sensitive to the superoxide radical-generating chemical, paraquat, the growth rates of PNG201 and *L. fermentum* BR11 were compared in growth medium supplemented with various concentrations of paraquat. It was found that *L. fermentum* BR11 cells grew well in paraquat concentrations as high as 90 mM (Fig. 3A). However, growth of PNG201 was slowed in the presence of 30 mM paraquat, and no growth was observed in 90 mM paraquat (Fig. 3B).

***L. fermentum* BR11 converts L-cystine into a free sulfhydryl compound, most of which is exported from the cell.** When *L. fermentum* BR11 cells were incubated with L-cystine and glucose, it was found that a relatively large amount of a sulfhydryl compound was exported from the cells (Table 1). This finding is consistent with the decrease in intracellular ^{14}C observed between the 3- and 5-min time points of the L-[^{14}C]cystine uptake assays (Fig. 2). In contrast to *L. fermentum* BR11, PNG201 was significantly impaired in its ability to export any sulfhydryl compounds when incubated with L-cystine and glucose (Table 1). Also during this incubation, the level of intracellular sulfhydryl compounds in *L. fermentum* BR11 increased so that it was significantly greater than that of PNG201 (Table 1). Examination of the normal intracellular sulfhydryl levels of *L. fermentum* BR11 and PNG201 cells which were not incubated with L-cystine revealed no significant difference (2.5 μmol of sulfhydryl/g [dry weight]).

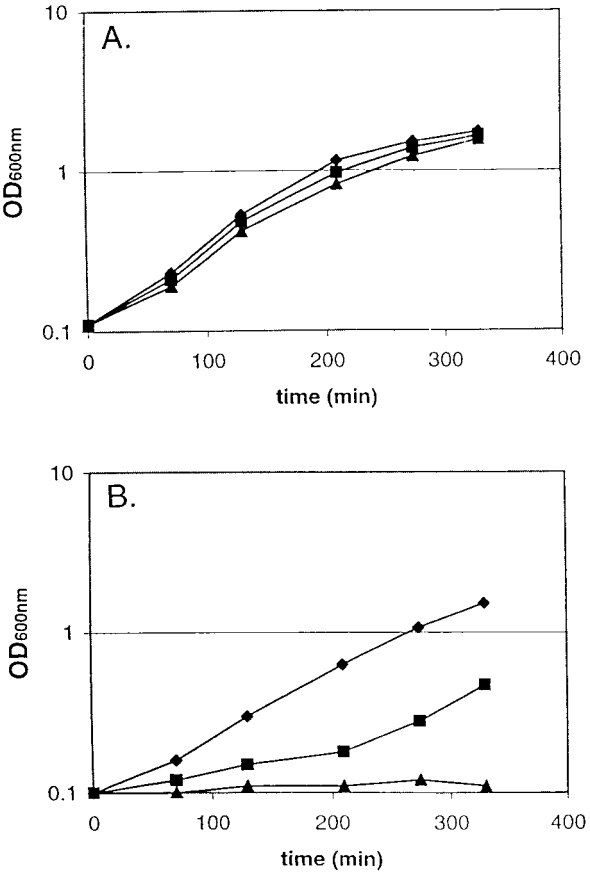

FIG. 3. Effect of paraquat on the growth of *L. fermentum* BR11 and PNG201. Growth of *L. fermentum* BR11 (A) and PNG201 (B) in the absence of paraquat (diamonds) or the presence of 30 mM (squares) or 90 mM (triangles) paraquat.

BspA does not mediate the binding of *L. fermentum* BR11 cells to type I collagen, fibronectin, or laminin. BspA has high similarity (88.6% amino acid identity) with the collagen binding protein, Cnb, from *L. reuteri* (28). To determine if BspA confers upon *L. fermentum* BR11 cells the ability to bind several ECM proteins, we developed a whole-cell enzyme-linked immunosorbent assay. It was found that *L. fermentum* BR11 cells did not adhere to immobilized type I collagen or laminin compared to the negative control skim milk proteins (Fig. 4). *L. fermentum* BR11 cells did adhere to immobilized fibronectin; however, PNG201 cells were not significantly reduced in their binding to immobilized fibronectin (Fig. 4).

TABLE 1. Distribution of sulfhydryls when *L. fermentum* BR11 and PNG201 cells were incubated with L-cystine (100 nmol)

L. fermentum strain	Mean ± SD distribution of sulfhydryls (nmol) (n = 3)	
	Extracellular	Intracellular[a]
BR11	45.2 ± 1.8	2.49 ± 0.23
PNG201	0.7 ± 0.3	0.94 ± 0.12

[a] When cells were not incubated with L-cystine, the normal level of intracellular sulfhydryls was 0.78 ± 0.12 nmol for *L. fermentum* BR11 and 0.69 ± 0.12 nmol for *L. fermentum* PNG201.

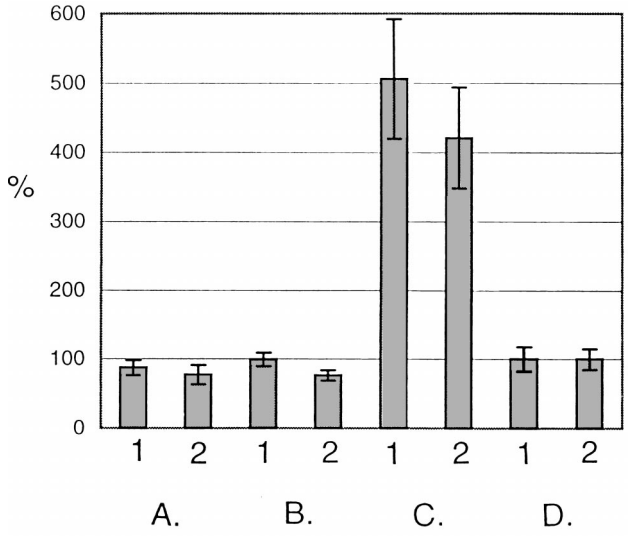

FIG. 4. Adherence of *L. fermentum* BR11 and PNG201 to immobilized type I collagen, laminin, and fibronectin. *L. fermentum* BR11 (1) or PNG201 (2) was added to wells coated with either type I collagen (A), laminin (B), fibronectin (C), or skim milk proteins (negative control) (D). Binding ability of cells to ECM proteins is plotted as percentages, compared to the binding to skim milk protein blocker, which was set at 100%. The assays were performed in quadruplicate, and the results (mean ± standard deviation) are from a representative experiment of three experiments.

DISCUSSION

On the basis of sequence similarities, BspA is a member of family III of the solute binding proteins as defined by Tam and Saier (33). It was therefore hypothesized that, like other members of this family, BspA binds a polar amino acid and transfers it to a membrane-located translocation complex. Identification of the specific ligands is complicated by the apparent extensive duplication and divergence of the sequences that have occurred through evolution and the consequent likelihood that alignable sequences are paralogous rather than orthologous. Therefore, in an attempt to identify an ortholog, BspA was aligned with all of the known proteins encoded by the recently sequenced *E. coli* genome (3). It was found that BspA is most similar to FliY (22). FliY is relatively uncharacterized compared with well-studied family III members such as GlnH (24), HisJ (14), and LAO (13) solute binding proteins. Expression of FliY has been shown to be induced under sulfate starvation conditions (27), and purified FliY has been shown to bind L-cystine (5). As yet, FliY has not been shown to mediate L-cystine uptake in *E. coli. L. fermentum* BR11 and two different BspA-depleted mutants were tested for their abilities to take up L-cystine. Uptake of radiolabelled L-cystine in *L. fermentum* BR11 was shown to be dependent upon glucose, thus demonstrating that this amino acid is being imported into the cells rather than just being bound to the cell envelope. Both the *bspA* mutants were found to be defective in L-cystine uptake. One of these mutants (PNG201) was also tested for L-glutamine, L-histidine, and L-lysine uptake and was found to be normal compared to the parent strain. It was therefore concluded that BspA is required for normal cystine transport at the low (3 μM) substrate concentration used and that it is not significantly involved in the uptake of L-glutamine, L-histidine, or L-lysine. It appears highly likely that BspA is the L-cystine binding protein in this uptake system and that it interacts with products of the other open reading frames at the *bspA* locus. Assuming this is the case, it is somewhat surprising that the BspA ligand was able to be identified by means of sequence comparisons across such a wide phylogenetic gulf. It indicates that either the common ancestor of gram-positive bacteria and proteobacteria contained a cystine binding protein directly ancestral to BspA or that there may have been more recent horizontal gene transfer events.

As gram-positive bacteria do not possess an outer membrane, it is thought that solute binding proteins are anchored to cytoplasmic membrane lipids to prevent their loss to the environment and to maintain proximity to the membrane translocation complex (32, 33). However, BspA does not possess the amino-terminal LXXC lipoprotein signature sequence. Also, during the initial characterization of BspA, its amino-terminal sequence was determined, which indicated that the amino terminus was not blocked due to covalent modification and that its signal sequence is removed at the predicted cleavage site for a type I signal peptidase (35). It therefore appears likely that BspA is not covalently attached to the cytoplasmic membrane. These results combined with the evidence that BspA is involved in L-cystine uptake lead us to conclude that solute binding proteins from gram-positive bacteria do not necessarily have to be covalently anchored to the cytoplasmic membrane in order to interact with the membrane translocation complex. This is the first report of a native and functional solute binding protein from a gram-positive bacterium which is not a lipoprotein.

Two mechanisms may account for the ability of BspA to interact with a membrane-associated transport complex. Firstly, processing of the signal peptide of BspA may be slow, therefore resulting in an adequate proportion of BspA being anchored to the cytoplasmic membrane by its uncleaved signal peptide. Similarly, it has been shown that inefficient cleavage of a signal peptidase I cleavage site introduced into the glycine betaine binding lipoprotein from *Bacillus subtilis* allowed it to continue to function in solute uptake (18). This model is unlikely to be correct in the case of BspA, because Western blots of whole-cell lysates show only one BspA band (that of processed BspA), thus suggesting that appreciable quantities of the unprocessed form are not present (35 and data not shown). The alternative and more likely model is that some or all of fully processed BspA stays close enough to the cytoplasmic membrane after translocation to allow interaction with the transporter.

L-cystine has recently been shown to be an excellent substrate for the *L. fermentum* DT41 cystathionine γ-lyase (31). Interestingly, an open reading frame from *L. fermentum* BR11 most likely present in the same operon as *bspA* potentially encodes a cystathionine γ-lyase (35). Breakdown of L-cystine by this enzyme results in the production of a free sulfhydryl compound (thiocysteine), a keto acid compound (pyruvate), and ammonia (31, 38). It is reasonable to hypothesize that once L-cystine is imported into *L. fermentum* BR11 by the BspA transport system, it is then broken down by the cystathionine γ-lyase enzyme. We show that substantial amounts of a sulfhydryl compound is rapidly exported from *L. fermentum* BR11 cells when incubated with L-cystine and glucose. The identity of this compound is not yet known; however, it is probably either thiocysteine or another sulfhydryl compound derived from thiocysteine. It is generally accepted that sulfhydryl compounds are essential flavor components in many varieties of cheese (4, 9, 10). In particular, the low-molecular-weight sulfhydryl compounds hydrogen sulfide and methanethiol appear to be important in cheddar cheese flavor development (20, 21, 36). Therefore, it is possible that proteins similar to those encoded by the *bspA* locus are involved in the production of sulfhydryl compounds by lactic acid bacteria in cheeses.

An interesting phenotype of PNG201 is that it grows more slowly in the presence of oxygen on solid medium and is more sensitive to paraquat than its parent. Paraquat is able to pass through the cytoplasmic membrane and generate superoxide radicals inside the cell (12). Superoxide is an unavoidable natural by-product of aerobic metabolism and has been shown to damage DNA, proteins, and membranes (11, 16). We hypothesized that because PNG201 is unable to take up L-cystine, it is therefore unable to convert it to sulfhydryl compounds which may protect the cell against oxidative stress. Although there was no significant difference in the normal intracellular sulfhydryl content between *L. fermentum* BR11 and PNG201, we believe that the oxidation-sensitive phenotype of PNG201 is due to its inability to increase the flux of sulfhydryl compounds into and out of the cell under oxidative conditions. It is possible that the low-molecular-weight sulfhydryl compound produced from the degradation of L-cystine has a role in protecting *L. fermentum* BR11 against oxidative stress. Similarly, it has been hypothesized that low-molecular-weight sulfhydryl compounds have a protective role against oxidative stress in the gram-positive streptomycetes (23).

The level of the intracellular sulfhydryl compound generated from L-cystine by *L. fermentum* BR11 appears to be tightly regulated, as only 4% of the total increase in sulfhydryls generated from L-cystine in 1 h remained intracellular, while the remainder was exported. In contrast, it has been reported for *Streptococcus mutans* that 60 to 80% of the total increase in sulfhydryls generated from L-cystine was intracellular (34). Why *L. fermentum* BR11 exports most of the sulfhydryl compound it generates from L-cystine is unknown; however, it may be to create reducing conditions in its immediate external environment, thus excluding aerobic bacteria and protecting against attack from oxidizing agents and electrophiles (6, 34). Export of sulfhydryls by lactic acid bacteria has been observed by a number of other researchers (7, 34). At first glance, the notion that a compound in its oxidized form (cystine) can contribute to resistance to oxidative stress appears odd. However, one interesting property of sulfur amino acid lyases is that they yield free sulfhydryl groups without consuming reducing equivalents. Presumably the reducing power comes from the replacement of an amine with a ketone inherent in the lyase reaction. If our model is correct, one ultimate source of reducing power for protection against oxidative stress is the amine group on a cystine molecule.

A number of previously described adhesins from the closely related streptococci have recently been shown to function in solute uptake (17, 19). Interestingly, BspA has homology to the collagen binding protein, Cnb, from *L. reuteri* (28) and the PEB1 adhesin from *C. jejuni* (25). Cnb has been shown to bind type I collagen by an affinity ligand blot procedure (1, 28). We have shown that semipurified BspA extracted from *L. fermentum* BR11 with 5 M LiCl binds type I collagen as well as fibronectin and laminin (data not shown). However, we did not find any evidence that BspA mediates the binding of *L. fermentum* BR11 cells to any of these immobilized ECM proteins. Although our experiments do not support the notion that BspA is an adhesin, this possibility is very difficult to rule this out, as only a small number of potential receptors have been tested.

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