

Cystathionine γ -Lyase Is a Component of Cystine-Mediated Oxidative Defense in *Lactobacillus reuteri* BR11[∇]

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Lactobacillus reuteri BR11 possesses a novel mechanism of oxidative defense involving an abundant cystine ABC transporter encoded by the *cyuABC* gene cluster. Large amounts of thiols, including H₂S, are secreted upon cystine uptake by the CyuC transporter. A cystathionine γ -lyase (*cgl*) gene is cotranscribed with the *cyu* genes in several *L. reuteri* strains and was hypothesized to participate in cystine-mediated oxidative defense by producing reducing equivalents. This hypothesis was tested with *L. reuteri* BR11 by constructing a *cgl* mutant (PNG901) and comparing it to a similarly constructed *cyuC* mutant (PNG902). Although Cgl was required for H₂S production from cystine, it was not crucial for oxidative defense in de Mann-Rogosa-Sharpe medium, in contrast to CyuC, whose inactivation resulted in lag-phase arrest in aerated cultures. The importance of Cgl in oxidative defense was seen only in the presence of hemin, which poses severe oxidative stress. The growth defects in aerated cultures of both mutants were alleviated by supplementation with cysteine (and cystine in the *cgl* mutant) but not methionine, with the *cyuC* mutant showing a much higher concentration requirement. We conclude that *L. reuteri* BR11 requires a high concentration of exogenous cysteine/cystine to grow optimally under aerobic conditions. This requirement is fulfilled by the abundant CyuC transporter, which has probably arisen due to the broad substrate specificity of Cgl, resulting in a futile pathway which degrades cystine taken up by the CyuC transporter to H₂S. Cgl plays a secondary role in oxidative defense by its well-documented function of cysteine biosynthesis.

Lactic acid bacteria (LAB) are known for their complex nutritional requirements and low tolerance for oxygen. Due to their lack of a heme biosynthesis pathway, LAB do not express fully functional catalases, but they have alternative mechanisms for oxidative defense. These include manganese-dependent pseudocatalase (25), superoxide dismutase (3, 6, 39, 50, 56), the alkyl hydroperoxide reductase system (15), and regulation of intracellular iron pool by Dpr (Dps-like peroxide resistance) (53). However, large gaps remain in the understanding of oxidative defense strategies in LAB, especially lactobacilli.

Lactobacillus reuteri is a member of the normal microbiota of mammalian mucosal surfaces, mainly intestinal tracts. The model organism of this study, *L. reuteri* BR11, was isolated from the vaginal tract of a guinea pig (36). Our group has previously identified a novel mechanism of oxidative defense in *L. reuteri* BR11 dependent on an L-cystine ABC uptake transporter encoded by the *cyuABC* gene cluster (*cyu* for cystine uptake) (Fig. 1) (45, 47). CyuC (formerly BspA) is a high-affinity L-cystine-binding protein that is an abundant component of the cell envelope (19). It is present at approximately 10⁵ molecules per cell, which is comparable to the abundance of S-layer subunits. Its abundance has led homologs in other strains of *L. reuteri* to be identified as adhesins or biosurfactant

compounds and named variously as Cnb (35), MapA (14), and p29 (11). *L. reuteri* rapidly transports cystine and converts it to a thiol(s), which is, at least in part, secreted from the cell in a process requiring CyuC (47). A *cyuC*-deficient mutant was found to be more sensitive to oxidative stress due to overproduction of hydrogen peroxide in the presence of oxygen (18). It was therefore concluded that the CyuC transporter plays a role in oxidative defense of *L. reuteri* BR11 by deriving thiols from exogenous cystine. The abundance of the CyuC transporter suggests that this transporter is highly significant for the physiology of *L. reuteri* BR11. This prompted us to further investigate the mechanism of CyuC-mediated oxidative defense.

Cystathionine lyases of LAB have been widely studied in the context of cheese flavor development (4, 5, 20, 28, 43). However, there are few studies about the physiological roles of cystathionine lyases of LAB. A partially sequenced homolog of a cystathionine γ -lyase (*cgl*) gene is located immediately upstream of the *cyu* gene cluster (45); therefore, this *cgl* gene was predicted to be cotranscribed with and functionally related to the *cyu* genes. Although Cgl is well known for its role in cysteine biosynthesis from cystathionine in the reverse transsulfuration pathway (17, 41, 44, 49, 52), cystathionine lyases have broad substrate specificities, and studies have shown that the Cgl proteins of *L. reuteri* and *Lactobacillus fermentum* are highly active against cystine and cysteine (5, 43). In addition, sulfur amino acid lyases are unique in that they are able to produce free thiols without consuming reducing equivalents (47). Therefore, it is reasonable to hypothesize that Cgl works in concert with the CyuC transporter in oxidative defense by cleaving cystine into thiols that act as reductants for thiol

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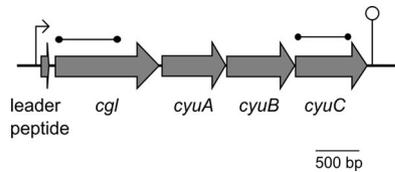


FIG. 1. *cyu* operon of *L. reuteri* BR11. The gray arrows indicate open reading frames, the right angle arrow denotes the promoter, and the lollipop denotes the terminator. The dumbbells indicate the regions of the *cgl* and *cyuC* genes that were used in constructing the mutants by homologous recombination. Leader peptide, cysteine-rich leader peptide involved in transcription attenuation; *cgl*, cystathionine γ -lyase; *cyuA*, membrane-spanning protein; *cyuB*, ATP-binding protein; *cyuC*, cystine-binding protein.

peroxidases (18, 47). Alternatively, thiols may reduce hydrogen peroxide nonenzymatically, as shown in the spontaneous reaction between sulfides and hydrogen peroxide (42).

The aim of this study was to clarify the role of *L. reuteri* BR11 Cgl in the CyuC-dependent oxidative defense mechanism. This was done by constructing and examining a mutant defective in Cgl activity. The central hypothesis was that an important role for Cgl is to generate reducing equivalents which can be coupled to oxidative defense. During the course of this study, it was found that although this hypothesis is in general supported by the data, the primary contribution of Cgl to oxidative defense is through cysteine biosynthesis. The production of other thiols, such as H_2S , as a result of low Cgl substrate specificity appears to represent a loss of reducing power available to the cell and to be the basis of a very high cysteine or cystine concentration requirement for optimal growth of *L. reuteri* BR11 in the presence of oxygen.

MATERIALS AND METHODS

Chemicals, enzymes, and kits. Unless otherwise stated, chemicals were purchased from Sigma (Castle Hill, NSW, Australia), and enzymes and kits for molecular biology manipulations were purchased from Roche (Castle Hill, NSW, Australia).

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Unless otherwise stated, *L. reuteri* BR11 and its derivatives were grown in de Mann-Rogosa-Sharpe (MRS) medium (Oxoid, Adelaide, SA, Australia) at 37°C under anaerobic conditions; agar plates were incubated in an AnaeroJar (Oxoid) containing an Anaerogen sachet (Oxoid); liquid cultures had minimal headspace and were incubated standing. Where required, BR11 derivatives were grown in the presence of 10 μ g/ml of erythromycin (Em) and chloramphenicol (Cm) when the antibiotics were used individually. The concentrations of Em and Cm were reduced to 5 μ g/ml and 7.5 μ g/ml, respectively, when the antibiotics were used in combination. For the *Escherichia coli* strains used in this study, Em, Cm, and kanamycin were applied at concentrations of 150 μ g/ml, 15 μ g/ml, and 40 μ g/ml, respectively.

Molecular cloning and transformations. Unless otherwise stated, molecular cloning procedures were carried out according to Sambrook and Russell (38). PCR was carried out using the Expand Long Template PCR system, and PCR products were purified with a High Pure PCR product purification kit. Templates for PCR amplification of genetic components of BR11 and its derivatives were cell extracts prepared as described below. Competent *E. coli* NCK1393 (Table 1) cells were prepared using a rubidium chloride method (33) modified from that of Hanahan (13) and transformed with the heat shock method (38). Minipreps were done using a QIAprep Spin miniprep kit (Qiagen, Doncaster, VIC, Australia). BR11 was transformed by electroporation as described previously (47), except that the plates were incubated at 35°C in the presence of Em and Cm. Sequencing was performed by the Australian Genome Research Facility (Brisbane, QLD, Australia).

Preparation of cell extracts. For PCR, cell extracts were prepared from 50-ml cultures at stationary phase (optical density [OD] between 1.8 and 2). The cells were harvested by centrifugation at 4,000 \times g for 10 min at 4°C, washed twice with TE (Tris-EDTA) buffer, pH 8, and resuspended in 1.5 ml of the same buffer. Cell suspensions were transferred to sealed 2-ml cryovials containing 0.5 ml of 0.1-mm zirconia-silica beads (DainTree Scientific, St. Helens, TAS, Australia) and homogenized in Mini-Beadbeater-8 (DainTree Scientific) at the highest speed for three cycles of 1 min each, with 1 min of cooling on ice between each homogenization. This was followed by centrifugation at 18,000 \times g for 5 min at

TABLE 1. Plasmids and bacterial strains used in this study

Plasmid or strain	Relevant properties	Reference or source
Plasmids		
pGh9:ISS1	<i>ori</i> ⁺ of pWV01; Em ^r of pIL253	31
pGh9	pGh9:ISS1 with EcoRI-SalI deletion to remove ISS1 element	This work
pNZ123	Heterogramic replicon of pSH71; Cm ^r of pC194	7
pPNG904	pGh9 ligated to EcoRI-SalI fragment of pNZ123 containing Cm ^r	This work
pPNG905	pPNG904 with BamHI deletion to remove Em ^r ; helper plasmid which provides <i>repA</i> in trans for pORI28	This work
pORI28	<i>ori</i> ⁺ of pWV01; no <i>repA</i> ; Em ^r of pUC19E	29
pPNG901	pORI28 with a 732-bp internal fragment of <i>cgl</i>	This work
pPNG902	pORI28 with a 621-bp internal fragment of <i>cyuC</i>	This work
pPNG903	pORI28 with a 729-bp internal fragment of <i>mlp</i>	This work
Strains		
<i>L. reuteri</i>		
BR11	Wild-type guinea pig vaginal isolate; Em ^s	36
PNG201	BR11 with pPNG201 integrated into the genome; <i>cyuC</i> deficient; Em ^r	47
PNG901	BR11 with pPNG901 integrated into the genome; <i>cgl</i> deficient; Em ^r	This work
PNG902	BR11 with pPNG902 integrated into the genome; <i>cyuC</i> deficient; Em ^r	This work
PNG903	BR11 with pPNG903 integrated into the genome; <i>mlp</i> deficient; Em ^r	This work
<i>E. coli</i>		
NCK1393	Also known as <i>E. coli</i> EC1000; contains chromosomally integrated <i>repA</i> for replication of pORI28 and derivatives; Km ^r	Todd Klaenhammer; 29
NCK1609	NCK1393 carrying pORI28; Em ^r	Todd Klaenhammer; 29

TABLE 2. Primers used in this study

Primer name	Application(s)	Sequence (5'–3') ^a
est/lip-F	Amplification and sequencing of <i>cgl</i> upstream region	GAGTATGATGCTAATGTTCCAGG
cgl-diag-R	Amplification and sequencing of <i>cgl</i> upstream region; diagnostic primer for confirmation of pPNG901 integration into genome	CAAAGTCCGCATCTGCTTAG
cgl-F	<i>cgl</i> insert primer	AATCTAGATA <u>ACCAACA</u> ACGGGTGC TGTTTC
cgl-R	<i>cgl</i> insert primer	CGTGAATTCTTATTGATGAACCCGCAT CCGTG
cyuC-F	<i>cyuC</i> insert primer	AAATCTAGATA <u>AAACAGTTGC</u> CAGCATT AACACTAG
cyuC-R	<i>cyuC</i> insert primer	ACTGAATTCTTAAATCATCTTAAGGCC CTTAGTAC
mlp-F	<i>mlp</i> insert primer	GGATCTAGATAAGCAGATAGCACCAC AGCAAATG
mlp-R	<i>mlp</i> insert primer	AACGAATTCTTAACCAGTAACAGAAC CATTAAACG
cgl-diag-F	Diagnostic primer for confirmation of pPNG901 integration into genome	GCATAAGGCGCAAATTCAAGTAAG
cyuC-diag-F	Diagnostic primer for confirmation of pPNG902 integration into genome	GCTCCAGATAATCCTAATGAAC
cyuC-diag-R	Diagnostic primer for confirmation of pPNG902 integration into genome	CTAACAGTCCCATCCTGTTG
mlp-diag-F	Diagnostic primer for confirmation of pPNG903 integration into genome	CTGTTGGTGTAGCGTCTGTTTTG
mlp-diag-R	Diagnostic primer for confirmation of pPNG903 integration into genome	CATTGGAAGCTGCAATGTTTTG
pORI-Em-start-R	pORI28-specific primer for confirmation of plasmid integration into genome	GTTCTCGAGGAAGTGTGCTGATTAC ^b
pORI-Em-end-F	pORI28-specific primer for confirmation of plasmid integration into genome	TTCTCGAGACATGCAGGAATTGACG ^b
cgl_br11_DIGf	Amplification of <i>cgl</i> probe for Northern blot analysis	TTCTCGTACTGGTAATCCAACG
cgl_br11_DIGr	Amplification of <i>cgl</i> probe for Northern blot analysis	ATGCTAATTGTTCCGCAATCTC
cyuC_br11_DIGf	Amplification of <i>cyuC</i> probe for Northern blot analysis	ATCTTCGGCAGTAAATTCAGAG
cyuC_br11_DIGr	Amplification of <i>cyuC</i> probe for Northern blot analysis	GTGAACAAAAACCGCCAAA

^a Restriction sites are bolded, and stop codons are underlined.

^b The restriction site was introduced for other experiments.

4°C to remove the beads. The supernatant was collected as the cell extract and stored at –20°C. Cell extracts for enzymatic assays were prepared in the same way, except that log-phase cells (OD between 0.5 and 1) were used, and the washing and resuspension buffer was 50 mM potassium phosphate buffer, pH 7.2.

Construction of helper plasmid pPNG905. The plasmids used in this study are listed in Table 1. pTRK699 (37), kindly provided by Todd Klaenhammer, was used initially to construct the mutants, but the attempt was unsuccessful. Therefore, we constructed pPNG905, an equivalent of pVE6007 which was used as the helper plasmid in constructing mutants of *L. reuteri* 100-23 (51). First, pPNG904 was constructed by replacing the *ISS1* element of pGh9:ISS1 with an EcoRI-SalI fragment of pNZ123 containing the Cm marker. Thus, this plasmid contains both Em and Cm markers. In order to produce a pVE6007 equivalent, the Em marker of pPNG904 was removed by digestion with BamHI and then ligated with T4 DNA ligase. Purified plasmid was obtained from *E. coli* NCK1393 transformed with the ligation and selected with Cm at 30°C.

Construction of the PNG901, PNG902, and PNG903 strains (*cgl*, *cyuC*, and *mlp* mutants). The mutants were constructed using single crossover insertional mutagenesis based on the method of Walter et al. (51). An internal fragment of each of the *cgl*, *cyuC*, and *mlp* genes was amplified by PCR using primers listed in Table 2 (Fig. 1). Each insert was digested with XbaI and EcoRI and ligated with similarly digested pORI28 (Table 1). The ligation reaction mixtures were transformed into *E. coli* NCK1393 to yield recombinant plasmids, the inserts of which were checked by sequencing before being transformed into BR11 containing the pPNG904 helper plasmid. Transformants were selected by incubation at 35°C for 48 h in the presence of Em and Cm and picked into liquid MRS to grow to stationary phase. Fifty microliters of this culture was transferred to 10 ml of fresh MRS and incubated at 44°C for 8 h. Dilutions of this culture were spread onto MRS plates containing Em and incubated at 44°C overnight. Colonies were replica plated onto MRS agar with Em and MRS agar with Cm, the latter to ensure that the helper plasmid was absent and thus that Em resistance was

brought about by integration of the plasmid into the genome. Em^r Cm^s colonies were regarded as putative integrants, and integration was confirmed by PCR of cell extracts with a combination of diagnostic primers that flank the insert and plasmid-specific primers (Table 2).

Surface protein extraction and SDS-PAGE analysis of surface proteins. Surface proteins were extracted using a procedure described previously (45, 47), with minor modifications. Briefly, 10 ml of stationary-phase cells was harvested by centrifugation at 4,000 × *g* for 10 min. The cell pellet was resuspended in 150 μl of 5 M LiCl and incubated at room temperature for 20 min and then centrifuged at 18,000 × *g* for 1 min. One hundred microliters of the supernatant, which contained the extracted surface proteins, was collected. The extract was mixed with an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (100 mM Tris-HCl, pH 7.0, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and run on a polyacrylamide (Bio-Rad, Gladesville, NSW, Australia) minigel (12% resolving and 5% stacking) prepared as described previously (26). The gel was run at 180 V for 60 min and stained with Coomassie brilliant blue R overnight.

Northern blot analysis. cDNA probes labeled with digoxigenin (DIG) were generated by direct PCR labeling of the amplicons with the following deoxynucleoside triphosphate concentrations: 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 190 μM dTTP, and 10 μM DIG-dUTP. A 530-bp probe of *cgl* and a 577-bp probe of *cyuC* were generated using primers listed in Table 2. Probes were analyzed with a 1.2% agarose gel and purified using a QIAquick gel extraction kit (Qiagen). Total RNA was isolated from cells harvested from an overnight culture of BR11 (10,000 × *g*, 10 min, 4°C) by using TRIzol reagent (Invitrogen, Mount Waverley, VIC, Australia) according to the manufacturer's instructions, with the addition of an extra phenol-chloroform extraction step. Twenty micrograms of total RNA was electrophoresed through a 1.0% agarose gel in the presence of 2.2 M formaldehyde. Equivalent loadings of samples were verified by ethidium bromide staining of the rRNA bands. The RNA was then

transferred onto a HyBond N+ membrane (GE Healthcare, Rydalmere, NSW, Australia) by using upward capillary transfer, followed by cross-linking with UV light. The membrane was subsequently hybridized for 16 h in DIG-Easy Hyb buffer (Roche) containing 25 ng/ml of probe at a hybridization temperature calculated according to the manufacturer's instructions. Hybridized bands were detected with a DIG nucleic acid detection kit.

Detection of extracellular thiols produced from cystine uptake. Thiols were detected using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as described previously (47). Overnight cultures were diluted and grown to log phase. The cells were then harvested, washed twice with potassium-phosphate-magnesium (KPM) buffer (47) at $4,000 \times g$ for 10 min at 4°C, and resuspended to an OD₆₀₀ of approximately 1. Five hundred microliters of cell suspension was then mixed with glucose (final concentration, 20 mM) and cystine (final concentration, 200 μM), and the reaction mixtures were incubated in a 37°C water bath for 1 hour. Subsequently, the reaction mixtures were centrifuged at $18,000 \times g$ for 1 min, 500 μl of the supernatant was mixed with 50 μl of 10 mM DTNB, and A₄₁₂ was measured. Negative controls without L-cystine and a control with no cells (reagent blank) were also tested. A standard curve was plotted with readings from a freshly prepared cysteine solution.

Measurement of C-S lyase activity toward cystathionine and cystine. The C-S lyase activity toward cystathionine and cysteine was determined by the amounts of thiols produced from these substrates, using DTNB. The protocol was largely based on that of Seefeldt and Weimer (40). One hundred microliters of cell extract prepared as described above was mixed with 1 mM of cystine or cystathionine, 20 μM of pyridoxal 5'-phosphate (PLP), and 0.28 mM DTNB in 50 mM potassium phosphate buffer (pH 7.2) in a final volume of 1 ml. Negative controls containing no substrate and reagent blanks containing no cell extracts were also tested. The reaction mixtures were incubated in a 37°C water bath for 1.5 h, and A₄₁₂ was measured. The protein content of the cell extracts was quantitated using a bicinchoninic acid protein assay kit (Sigma).

Detection of cysteine desulfhydrase activity. The protocol was largely similar to that of Yoshida et al. (57). Cell extracts prepared from log-phase cells, as for measurement of C-S lyase activity, were mixed with Tris-glycine native sample buffer, loaded onto a nondenaturing polyacrylamide gel (12% resolving and 5% stacking), and electrophoresed at 4°C at 20 mA for 2 h. After electrophoresis, the gel was incubated in 10 ml of visualizing solution in a 37°C hybridization oven with gentle agitation to detect a black band of sulfide precipitate. The visualizing solution contained 100 mM of triethanolamine-HCl (pH 7.6), 10 μM of PLP, 0.5 mM of bismuth(III) subnitrate, 10 mM of EDTA, 1% of Triton X-100, and 5 mM of L-cysteine.

Growth experiments. Overnight cultures were grown to log phase and transferred to fresh MRS to result in a starting OD₆₀₀ of about 1.2. Aerobic cultures occupied 1/10 of the volume of the tube and were aerated at 220 rpm. OD₆₀₀ was recorded hourly for 8 to 10 h, and a final reading was sometimes also taken the next day, after 23 or 24 h. When required, MRS was supplemented with cysteine, cystine, methionine, dithiothreitol (DTT), bovine liver catalase, or hemin at concentrations indicated in the text. Water was used as the solvent for preparing stock solutions of the supplements, except for cystine (0.5 M HCl), catalase (50 mM potassium phosphate buffer, pH 7), and hemin (50 mM NaOH). Supplements were prepared fresh on the day of the experiment and filter sterilized with 0.2-μm syringe filters before being added into the culture media.

Detection of volatile sulfur compounds generated from cystine uptake. The volatile sulfur compounds were detected with a solid-phase microextraction fiber (Supelco; Sigma, Castle Hill, NSW, Australia) on a Varian CP-3800 gas chromatograph (Alderley, QLD, Australia) equipped with a pulsed-flame photometric detector. Log-phase cells were washed and resuspended as for detection of extracellular thiols by DTNB. Two milliliters of cell suspension was transferred to a gas chromatography (GC) vial and incubated with 5 mM of glucose and 50 μM of cystine at 37°C for 30 min. A StableFlex 85-μm Carboxen-polydimethylsiloxane fiber was then injected into the headspace of the vial and allowed to adsorb the volatiles for 15 min. After that, the fiber was thermally desorbed into the GC-pulsed-flame photometric detector injector at 250°C for 7 min and operated in the splitless mode. The injector temperature was set at 250°C and fitted with a narrow bore (0.75-mm inside diameter) inlet liner (Supelco). The analytes were separated with a CP-SIL 5 CB column (fused silica, 30-m by 0.32-mm inside diameter, 4-μm film thicknesses; Varian). The oven temperature was programmed as follows: 35°C for 2 min, followed by an increase at 15°C/min to 150°C and holding for 1 min. After that, the temperature was increased at 20°C/min to 250°C and held for 2 min. The total run time was 16 to 17 min. The detector parameters were as follows: temperature, 300°C; tube volt, 550 V; trigger level, 200 mV; sampling delay, 6 ms; sampling width, 20 ms; and gain factor, 2. The chemical standards used were hydrogen sulfide, methanethiol, carbonyl sulfide, dimethyl disulfide, dimethyl sulfide, dimethyl trisulfide, carbon

disulfide, dimethyl sulfoxide, and dimethyl sulfone. Hydrogen sulfide was generated as described previously (48). The carrier gases, helium, and instantaneous air had a flow rate of 2 ml/min.

Hydrogen peroxide measurements. Hydrogen peroxide was measured using the FOX (ferric-xylenol orange) method as described previously (9, 23, 27). A FOX reagent mixture containing 25 mM of sulfuric acid, 100 mM of sorbitol, 0.25 mM of ferrous ammonium sulfate, and 0.1 mM of xylenol orange was prepared. At each time point in the growth experiments, an aliquot of the culture was centrifuged at $18,000 \times g$ for 1 min, and 50 μl of the supernatant was mixed with 950 μl of the FOX reagent mixture. The reaction mixtures were incubated at room temperature in the dark for 30 min, and A₅₆₀ was measured. A standard curve was prepared with MRS containing 0 to 100 μM of hydrogen peroxide. The FOX reagent mixture and stock solutions of its components were all prepared fresh on the day of the assay.

Nucleotide sequence accession number. The sequence of the *cyu* operon containing the complete sequence of *cgl* and upstream regions is included in the updated GenBank entry U97348.

RESULTS

The *cyu* operon is highly conserved among *L. reuteri* strains.

The *cyu* genes and part of the upstream *cgl* gene of BR11 have previously been sequenced (GenBank accession no. U97348) (45). In order to obtain the complete sequence of *cgl* and the promoter, PCR was performed using the primers est-lip-F and *cgl*-diag-R (Table 2). The forward primer (est-lip-F) was designed to bind to a region upstream of *cgl* on the basis of homologs in *L. reuteri* 104R (AJ293860), F275 (NC_009513), and 100-23 (NZ_AAPZ00000000). This strategy yielded the complete sequence of *cgl* and, upstream of it, a short open reading frame and a putative promoter (Fig. 1). *Cgl* of BR11 belongs to the MetC family of cystathionine lyases. Multiple alignments of *Cgl* with all the homologous sequences of *L. reuteri* strains available in GenBank (the three mentioned above and strain ATCC 55730) revealed 94 to 95% identity at the amino acid level. The eight active site residues and the two residues for tetramer contact (32) are all conserved. MetC-type cystathionine lyases of *Lactobacillus* spp. have been reported to occur in *Lactobacillus fermentum* DT41 (43), *Lactobacillus helveticus* CNRZ 32 (28), and *Lactobacillus casei* FAM18168 (20). Sequences are available for only the last two, and they have identities of 65% and 45% and similarities of 81% and 67%, respectively, to *Cgl* of BR11 at the amino acid level. An unpublished study of *L. reuteri* 104R cited under GenBank accession no. AJ293860 found that expression of the *mapA* operon, which is homologous to the *cyu* operon, is regulated by transcription attenuation involving cysteine. The short open reading frame upstream of *cgl*, which contains three tandem cysteine codons, is likely to encode the leader peptide involved in this mechanism. The leader peptides of *L. reuteri* 104R, F275, and 100-23 are identical at both the amino acid and the nucleotide levels, and that of BR11 differs only at two amino acids. The three cysteine codons are conserved in all the strains examined, suggesting that regulation of the *cyu* operon by transcription attenuation is universal in *L. reuteri*. Analysis of the promoter region showed that the putative -35 and -10 boxes (TTGACA and TATGAT, respectively) are identical in all the strains examined. Based on sequence analysis, there is only one promoter upstream of *cgl* and no transcription terminator between *cgl* and *cyuA*. Therefore, it was hypothesized that *cgl* is cotranscribed with the *cyu* genes. This was confirmed by Northern blot analysis, in which probes derived from the *cyuC* and *cgl* genes each hybridized to a single band of approx-

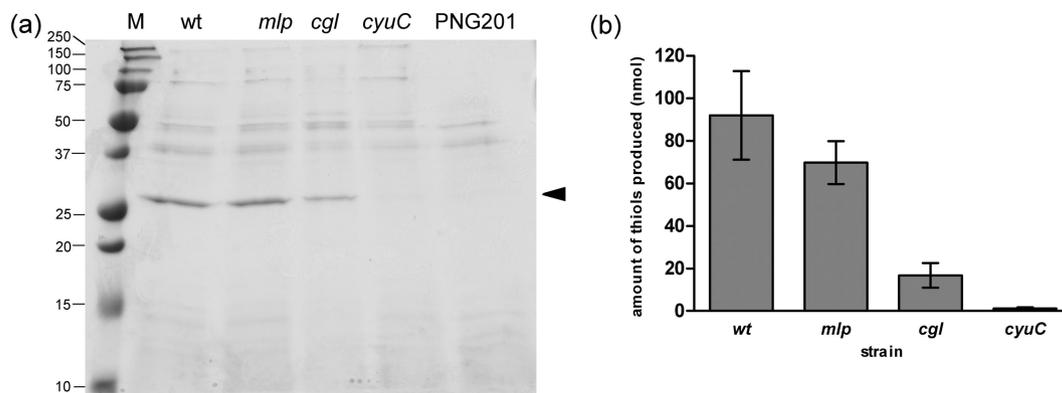


FIG. 2. PNG901 (*cgl* mutant) retains functional cystine uptake. (a) SDS-PAGE gel of LiCl extracts of surface proteins, stained with Coomassie brilliant blue R. M, protein marker, with the sizes of the marker bands in kDa indicated on the left; wt, wild-type BR11; *mlp*, PNG903; *cgl*, PNG901; *cyuC*, PNG902; PNG201, *cyuC* mutant constructed in previous study. The band corresponding to CyuC is indicated with the pointer on the right. (b) Thiols produced by whole cells upon cystine uptake detected by DTNB. The mean values for three independent experiments are shown. Error bars indicate standard deviations.

imately 3.5 kbp (data not shown). This is the expected size if *cgl* and the *cyu* genes constitute an operon.

PNG901, a single-crossover *cgl* mutant with functional cystine uptake, was constructed. The *cgl* gene in PNG901 was inactivated by single-crossover recombination using a homologous DNA fragment internal to *cgl* (Fig. 1). Since the integrated plasmid resulting from the single crossover was expected to inactivate the entire *cyu* operon, a *cyuC* mutant (PNG902) was constructed in the same way so as to obtain insight into Cgl function by comparing the effect of inactivating Cgl and the CyuC transporter with the effect of only inactivating the CyuC transporter. In order to control for any differences in phenotypes caused by the plasmid integration event itself, PNG903 was also constructed with the same approach. PNG903 was inactivated in the Mub-like protein (*mlp*) gene (46) on the basis of the previous observation that *mlp* deficiency does not confer any growth defect in BR11 (M. Turner, unpublished findings). Surprisingly, SDS-PAGE analysis of LiCl-extracted surface proteins showed that CyuC was still produced by PNG901 (Fig. 2a). Given that the Northern blot data from the wild type had provided no evidence for a transcription start site downstream of the *cgl* promoter, it was concluded that there is an active promoter in the integrated plasmid and that the CyuC transporter is functional in the *cgl* mutant. This is consistent with the findings by Leenhouts et al. that the P_{repC} promoter in pORI280, a derivative of pORI28, drives the expression of genes downstream of the target gene in a single-crossover mutant, thus resolving the problem of polar effects (29). The homologous inserts for constructing the three mutants in this study were all cloned into pORI28 in the orientation that allows transcription of downstream genes from the P_{repC} promoter.

If Cgl is the only enzyme that catabolizes exogenous cystine into thiols, PNG901 will not produce thiols upon cystine uptake. In order to test this hypothesis, whole cells were incubated with glucose and cystine, and the supernatant was reacted with DTNB to detect thiol production. As shown in Fig. 2b, PNG901 still retained the ability to produce thiols from cystine uptake, though the amount of thiols released was only about 18% of that produced by wild-type cells. This suggests

that Cgl is involved in thiol production from cystine uptake, but it is not the only cause of this reaction. It is difficult, however, to rule out the effect of a possibly lower level of expression of the CyuC transporter in PNG901. Therefore, subsequent experiments were carried out using cell extracts.

Cgl is required for thiol production from cystine in cell extracts. In order to further characterize the enzymatic activity of Cgl toward cystine, DTNB was reacted with cell extracts in the presence of cystine and PLP. Cystathionine was also used as a substrate for comparison. PNG901 had no activity against cystine, whereas PNG902 had activity similar to that of the wild type (Fig. 3a), which is consistent with the expectation that the *cgl* gene is intact in PNG902. This result confirms that Cgl cleaves cystine into thiols and that Cgl is the only enzyme in BR11 that mediates this reaction under the experimental conditions used.

It has previously been reported that the thiols secreted upon cystine uptake include H_2S (18). To study this further, bis-muth(III) subnitrate was used as an activity stain in a native PAGE analysis. Both cystine and cysteine were used as substrates in two separate native PAGE analyses in the presence of PLP. However, cystine precipitated out, most likely because it was not soluble at the pH of the assay buffer. Conversely, the native PAGE with cysteine revealed that PNG901 could not cleave cysteine into H_2S (Fig. 3b). Therefore, Cgl is the only enzyme in BR11 with cysteine desulfhydrase activity under these assay conditions.

In order to determine if Cgl is involved in H_2S production from cystine in whole cells, volatile sulfur compounds released upon cystine addition were detected by GC. Cells were incubated with cystine and glucose in a GC vial, and a solid-phase microextraction fiber was exposed to the headspace in the vial. The chromatograph of the wild type showed a prominent peak of H_2S , while PNG901 and PNG902 produced no detectable volatile sulfur compounds (data not shown). It was concluded that both CyuC and Cgl are required for the production of H_2S from cystine by BR11.

CyuC but not Cgl is crucial for growth under aerobic conditions. The contribution of Cgl to oxidative defense was tested using growth experiments with liquid MRS medium incubated

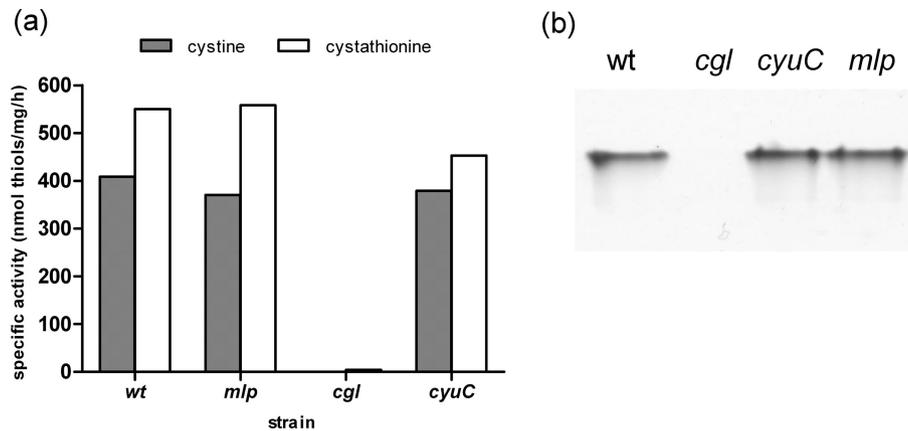


FIG. 3. Enzyme activity of Cgl against cystine and cystathionine. wt, wild-type BR11; *mlp*, PNG903; *cgl*, PNG901; *cyuC*, PNG902. (a) C-S lyase activity of cell extracts against cystine, expressed in terms of thiols produced. The activity against cystathionine was also tested for comparison. DTNB was used for thiol detection. (b) Native PAGE gel showing cysteine desulfhydrase activity of cell extracts visualized by the black precipitate resulting from reaction between hydrogen sulfide and bismuth(III) subnitrate.

under both anaerobic conditions (standing cultures) and aerobic conditions (aerated cultures). PNG903 had the same growth profiles as the wild type under all the conditions tested (data not shown), confirming that the plasmid integration event did not affect the growth of the mutants. Under anaerobic conditions, although all strains reached similar final ODs after 24 h of growth, PNG901 and PNG902 displayed growth defects in the form of longer generation times (Fig. 4a), indicating that Cgl and CyuC are important in maintaining basal cellular functions other than oxidative defense. These growth defects most likely reflect deficiencies in cysteine for protein biosynthesis. Under aerobic conditions, all strains grew to ODs lower than those observed under their respective anaerobic conditions (Fig. 4b). The growth rates of the wild type and PNG901 were similar to their respective growth rates under anaerobic conditions. Since PNG901 had an inherent growth defect even in the absence of oxygen (Fig. 4a), it was unclear whether its growth defect under aerobic conditions was due to increased sensitivity to oxidative stress. On the other hand, PNG902 displayed a remarkably severe growth defect under

aerobic conditions; it was arrested in lag phase (Fig. 4b). Although PNG902 also had an inherent growth defect under anaerobic conditions (Fig. 4a), the severity of the growth impairment in aerated cultures was sufficient evidence of its increased sensitivity to oxidative stress. These results show that what is essential for growth under aerobic conditions is cystine uptake, carried out by the CyuC transporter. The reactions catalyzed by Cgl, ranging from catabolism of cysteine and cystine into H_2S to biosynthesis of cysteine from methionine, are not crucial for oxidative defense.

The growth defect of PNG902 under aerobic conditions supports the results that we previously obtained with PNG201, a *cyuC*-deficient mutant constructed using a different plasmid and homologous gene fragment (47). However, the difference in severity of growth defects between the two mutants is anomalous (Fig. 4). Due to differences in the mutant construction methods, an intact copy of the *cyuC* gene is present in PNG201 but not in PNG901. This led us to believe that the phenotypes of the current *cyuC* mutant reflect more accurately the consequences of an inactivated *cyuC* gene. We speculate that *cyuC* in

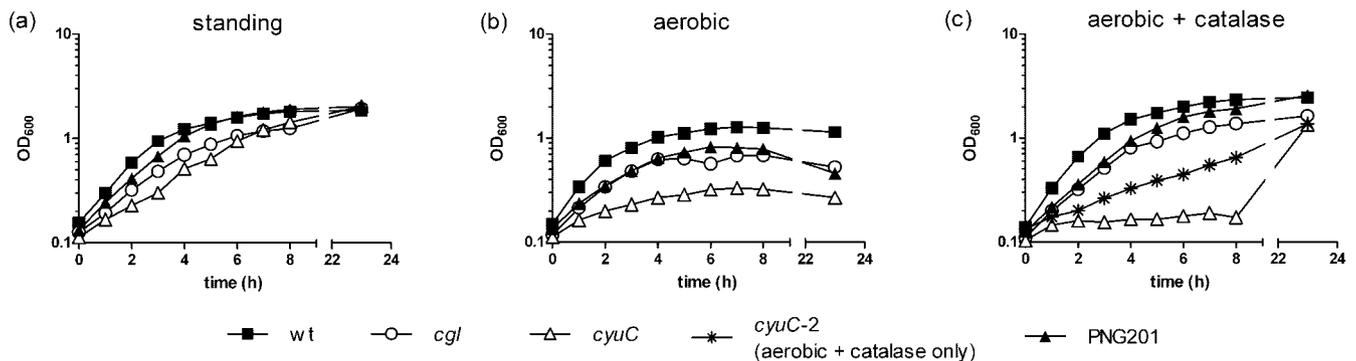


FIG. 4. Effects on growth of aeration and catalase. (a) Standing cultures, (b) aerated cultures, (c) aerated cultures supplemented with 800 U catalase. wt: BR11; *cgl*, PNG901; *cyuC* and *cyuC2*, PNG902; PNG201, *cyuC* mutant constructed in a previous study (47). The growth experiments were repeated independently at least three times, and representative results are shown here. Individual experiments yielded reproducible results under all conditions, except in the case of the aerobic growth of PNG902 in the presence of catalase, shown as *cyuC* and *cyuC-2*. Each mode of growth of PNG902 in the catalase-supplemented cultures was seen in at least two independent experiments. The curves of PNG903 (*mlp* mutant) overlap with those of the wild type and so are omitted in all the growth experiment graphs for the sake of clarity.

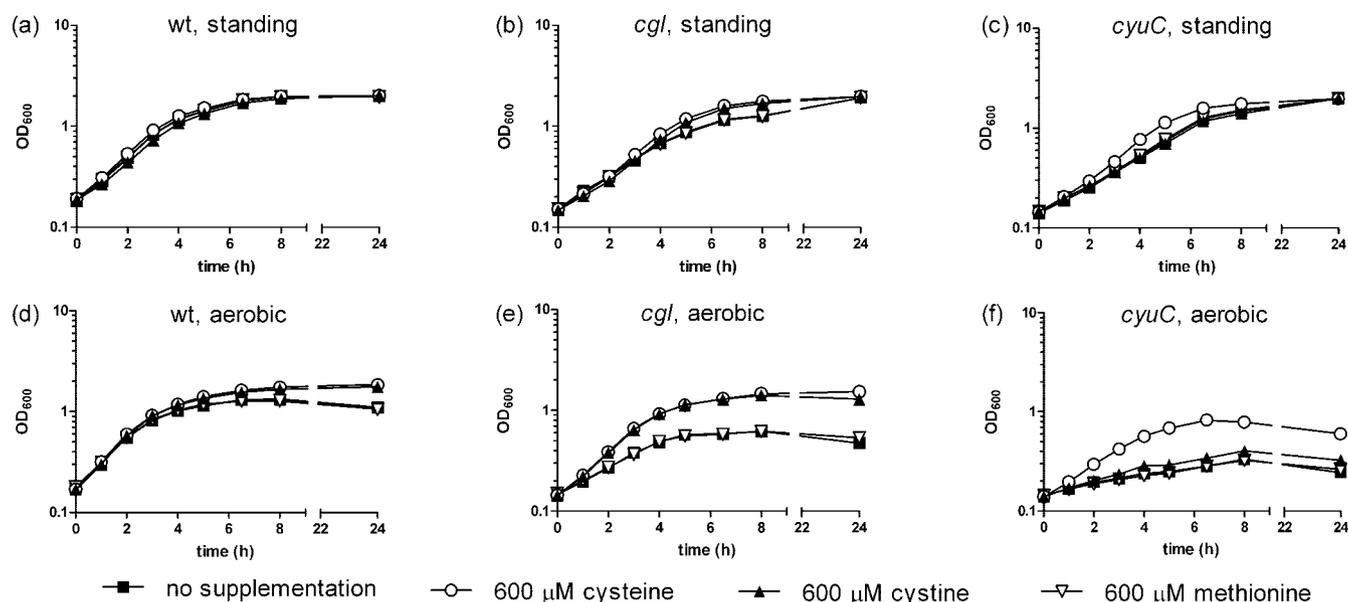


FIG. 5. Effects on growth of 600 μ M cysteine, cystine, and methionine. (a and d) BR11 in standing and aerated cultures, respectively; (b and e) PNG901 in standing and aerated cultures, respectively; (c and f) PNG902 in standing and aerated cultures, respectively.

PNG201 may not be fully inactivated, or that strain may have accumulated secondary mutations that compensate for the deleterious effects of the disrupted *cyuC*.

It has previously been reported that PNG201 is oxygen sensitive due to overproduction of H_2O_2 (18). The bases for this conclusion were that PNG201 enters stationary phase at a lower OD than BR11, the H_2O_2 concentrations in stationary phase are similar for PNG201 and BR11, and catalase addition increases the stationary-phase ODs of PNG201 and BR11 to the same values. Similar experiments were carried out on PNG901 and PNG902. The results for PNG201 were essentially as previously reported (18), with a lower stationary-phase OD under aerobic conditions (Fig. 4b) and at least partial elimination of this effect by 800 U catalase (Fig. 4c). BR11 and PNG901 responded similarly to catalase, with the ODs in stationary phase being higher and the growth curves resembling those seen under anaerobic conditions (Fig. 4c). It was difficult to obtain reproducible results for PNG902, because its growth defect in aerated cultures includes an extremely long and variable lag phase and very slow growth, neither of which was clearly affected by catalase (Fig. 4c). It was concluded that for all the strains except PNG902, H_2O_2 accumulation limits the stationary-phase OD under aerobic conditions. In contrast, PNG902 exhibits oxygen sensitivity that is independent of H_2O_2 accumulation.

Cysteine is protective against oxidative stress. In order to determine whether it is cysteine itself or other thiols, such as H_2S derived from cystine/cysteine, that are protective against oxidative stress, growth experiments in which anaerobic and aerobic cultures were supplemented with various concentrations of cystine and cysteine were performed. Under both anaerobic and aerobic conditions, the same concentrations of cystine and cysteine had the same effect in PNG901 (Fig. 5b and e and data not shown), thus confirming that the *CyuC* transporter is functional in this mutant. The interchangeability

of cysteine and cystine also applies to the wild type under aerobic conditions (Fig. 5d and data not shown). In PNG902, cystine had no effect (Fig. 5c and f and data not shown), whereas cysteine restored growth (Fig. 5c and f). This confirms that cystine uptake in PNG902 is abolished, while cysteine uptake is functional and mediated by a transporter other than the *CyuC* system.

Under anaerobic conditions, cysteine supplementation had no effect on the wild type (Fig. 6a), suggesting that the concentration of sulfur amino acids in MRS is already sufficient for the cysteine requirements of BR11 in MRS. On the other hand, a dose-dependent effect was apparent in both PNG901 and PNG902 (Fig. 6b and c). Growth was improved to match the level of the wild type when the cysteine concentration was 600 μ M or more. The alleviation of anaerobic growth defects of both PNG901 and PNG902 by cysteine supplementation suggests that both reverse transsulfuration (*Cgl*) and cystine uptake (*CyuC* transporter) are necessary to maintain adequate intracellular cysteine levels for protein biosynthesis.

Under aerobic conditions, cysteine improved the growth of the wild type slightly in the form of increasing the final OD (Fig. 6d), suggesting that cysteine is protective against oxidative stress and that under aerobic conditions, BR11 has a higher cysteine concentration requirement. A dose-dependent effect for cysteine was seen again in PNG901 and PNG902 in the aerobic cultures (Fig. 6e and f). The ability of cysteine to rescue the oxidatively stressed PNG902, whose cystine uptake function is abolished but whose cysteine uptake function remains intact, suggests that when wild-type BR11 is faced with oxidative stress, the protective agent derived from exogenous cysteine taken up by the *CyuC* transporter is cysteine. PNG902 clearly showed a much higher cysteine concentration requirement than PNG901. Among the concentrations tested, only 6 mM of cysteine was able to restore its growth to the wild-type level, while 600 μ M was already sufficient for PNG901. These

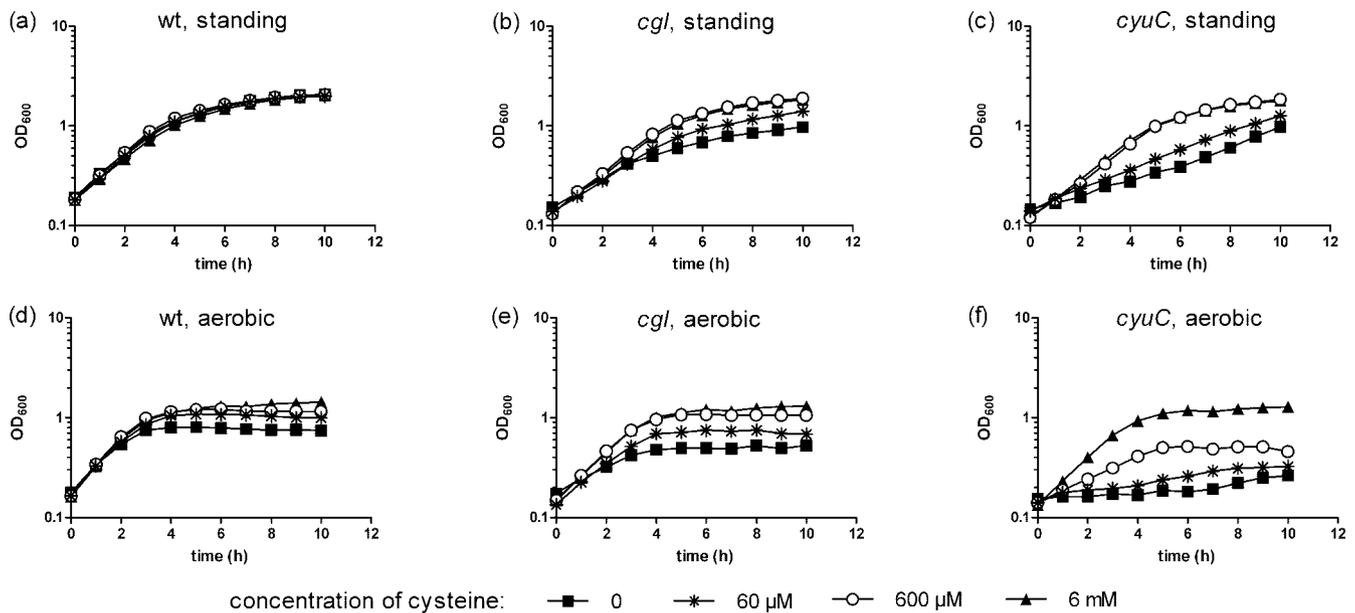


FIG. 6. Effects on growth of different cysteine concentrations. (a and d) BR11 in standing and aerated cultures, respectively; (b and e) PNG901 in standing and aerated cultures, respectively; (c and f) PNG902 in standing and aerated cultures, respectively.

results suggest that under aerobic conditions, cystine uptake is crucial in providing cysteine for oxidative defense. On the other hand, the similar requirement of PNG901 for cysteine under anaerobic and aerobic conditions suggests that its growth defect in aerated cultures is simply due to insufficient cysteine for protein biosynthesis.

Since methionine is the precursor of cysteine in the reverse transsulfuration pathway, we hypothesized that methionine would be able to improve the growth of PNG902 but not PNG901 because the former contains an intact *cgl* gene. Anaerobic cultures were supplemented with either 600 μM of methionine, cysteine, or cystine or 6 mM of methionine. No improvement in growth was seen in the methionine-supplemented cultures of any of the strains, even at a concentration as high as 6 mM, in contrast to the cultures supplemented with cysteine (in all strains) and cystine (the wild type and PNG901) (Fig. 5 and data not shown). This suggests that the methionine-to-cysteine conversion in BR11 is inefficient and/or rate limited or that the cells cannot transport methionine.

Cgl is important in protection against hemin-induced stress. Several species of LAB have been shown to be able to respire when heme is provided, and survival is better when cells are respiring than when they are fermenting under aerated conditions (8, 54). BR11 contains genes encoding cytochrome *bd* (data not shown), and the genome sequences of *L. reuteri* F275 and 100-23 include the majority of other genes that were reported to be necessary for respiration in LAB, although the menaquinone biosynthetic pathway appears incomplete. Nevertheless, there was a possibility that the improvement in growth in the catalase-supplemented cultures (Fig. 4c) was simply due to cells utilizing heme in the catalase for respiration. In order to confirm that the rescue of aerobic cultures by catalase was specifically due to reduction of H₂O₂, cells grown anaerobically and aerobically were challenged with different concentrations of hemin: 0.5, 4, 8, and 16 μM (the heme

content of the amount of catalase used is 0.47 μM). Under anaerobic conditions, both PNG901 and PNG902 showed sensitivity to hemin similar to that of the wild type (data not shown). However, under aerobic conditions, a much higher sensitivity to hemin was seen in both mutants (Fig. 7a, b, and c). The lag-phase arrest of PNG902 under aerobic conditions makes it difficult to interpret the effect of hemin (Fig. 7c), but the difference in OD between PNG901 cells in the absence of hemin and those challenged with 0.5 μM of hemin is striking (Fig. 7b). These results indicate that the stress resulting from hemin occurs only under aerated conditions, which agrees with previous studies showing the involvement of heme in oxidative stress (1, 2, 10). Therefore, the rescue brought about by catalase supplementation was not due to a shift to respiration by BR11. In fact, these preliminary results suggest that BR11 is not able to respire in aerated MRS medium when heme is provided, possibly because a quinone source is also needed (54). Fortunately, they revealed that Cgl is important for protection against heme stress.

Gaudu et al. reported that both cysteine and DTT alleviated heme-induced toxicity in a *ccpA* mutant of *Lactococcus lactis* (12). In order to determine whether the heme sensitivity of PNG901 is due to a deficiency in thiols in general or in cysteine specifically, cells grown aerobically in the presence of hemin were supplemented with cysteine, cystine, methionine, or DTT (Fig. 7d, e, and f). In PNG901, only cysteine or cystine alleviated the growth defects caused by hemin (Fig. 7e). In PNG902, 6 mM of cysteine was required to improve growth to the level of PNG901 brought about by 1 mM of cysteine. DTT restored the growth of PNG902 to the level of cells grown in the absence of hemin (Fig. 7f). Methionine had no effect on the wild type or the mutants. These results suggest that hemin toxicity involves deprivation of cysteine, not thiols in general, and that the role of *cgl* in protection against heme stress is biosynthesis of cysteine.

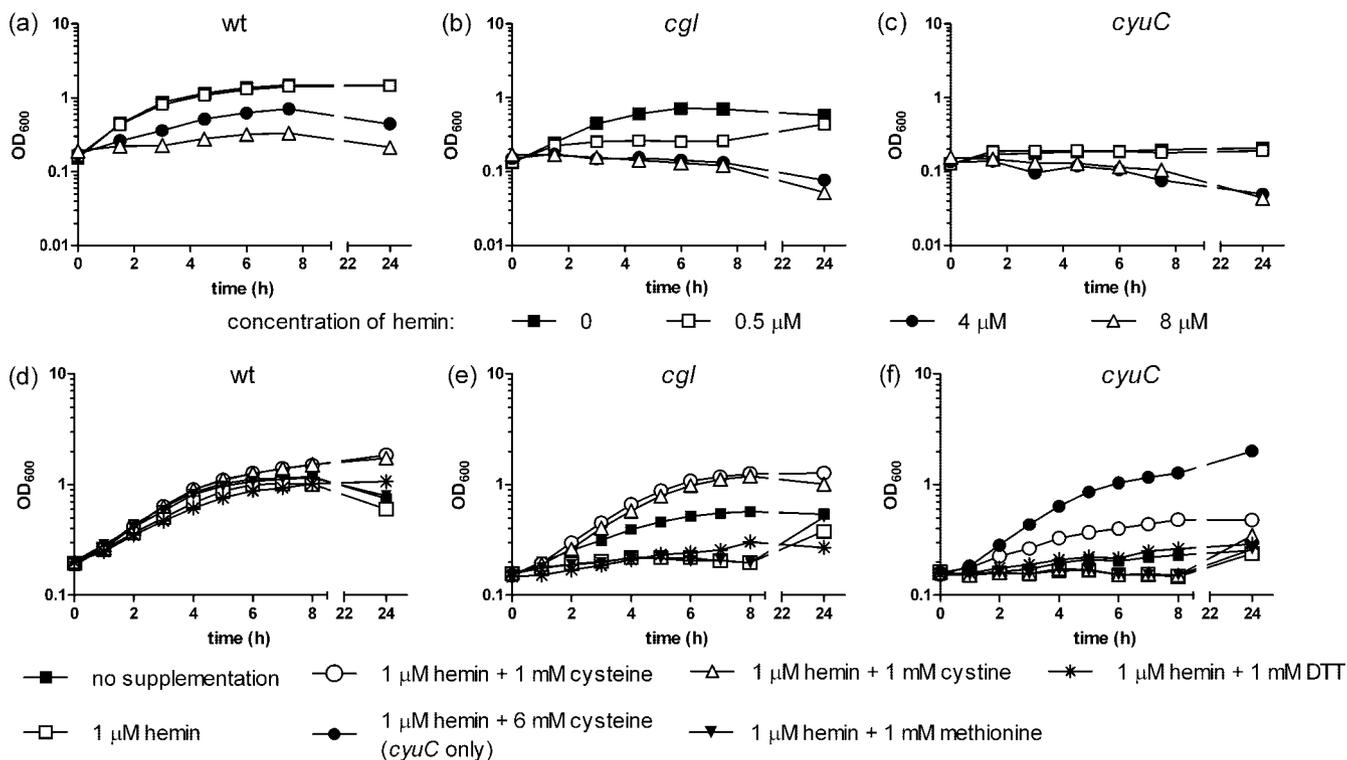


FIG. 7. Hemin challenge and rescue with cysteine/cystine in aerated cultures. wt, wild-type BR11; *cgl*, PNG901; *cyuC*, PNG902. (a to c) Growth in the presence of various concentrations of hemin. Concentrations of hemin used are indicated in the legend. (d to f) Effect of sulfur amino acids and DTT on alleviation of hemin stress. The cells were challenged with 1 μM of hemin. Concentration and type of supplement used are indicated in the legend.

DISCUSSION

This study is a follow-up to the discovery of a novel oxidative defense pathway in bacteria that is dependent on cystine uptake. BR11 possesses a highly expressed L-cystine ABC-type transporter that takes up cystine, which is converted into thiols that are subsequently secreted into the extracellular environment. Our hypothesis was that the cotranscribed Cgl protein is essential in the formation of these thiols, which protect BR11 against oxidative stress enzymatically by acting as a reductant for thiol peroxidase(s) or nonenzymatically by directly reducing reactive oxygen species. The results of this study, however, indicate that this model is incorrect in important aspects. There is a specific requirement for cysteine for oxidative defense, and other thiols, such as H₂S, cannot substitute for cysteine in this role. The key result is that the *cgl* mutant (PNG901) is completely unable to produce H₂S but is much less sensitive to oxygen than the *cyuC* mutant (PNG902), which cannot transport cystine. This disproves the conjecture that a major role for Cgl is the generation of reducing equivalents. Rather, despite its lack of substrate specificity, the clear role of Cgl is its commonly accepted role of cysteine biosynthesis.

Some aspects of the sulfur economy of BR11 are challenging to reveal, and there are still anomalies remaining. One observation that is difficult to explain is that the *cyuC* mutant and the *cgl* mutant have similar growth defects under anaerobic conditions and that these defects can be complemented with similar concentrations of cysteine. However, under aerobic condi-

tions, the effect of inactivating *cyuC* is much greater than the effect of inactivating *cgl*. These data suggest that in anaerobic MRS culture, CyuC and Cgl make similar contributions to the intracellular cysteine pool, while under aerobic conditions, the contribution of CyuC is much greater than that of Cgl. We have considered invoking separate intracellular pools of cysteine, one derived from biosynthesis and used in protein synthesis and one derived from uptake and used in oxidative defense. However, the demonstration that the *cgl* mutant is also compromised in oxidative defense, albeit slightly, such that it is manifested only as heme sensitivity, leads to a more conservative model. That is, the required intracellular concentration of cysteine is higher under aerobic conditions than under anaerobic conditions. Cgl not only synthesizes cysteine, it also degrades it to H₂S. It is inevitable that the higher the cysteine concentration in the cell, the greater the bias toward cysteine breakdown as opposed to cysteine synthesis. Therefore, achieving high intracellular concentrations may be difficult and require a very active transporter. It appears that *L. reuteri* has evolved a futile or wasteful pathway in which the cell actively acquires cystine or cysteine by both synthesis and active transport and rapidly loses it due to Cgl-mediated breakdown. It is noteworthy that the *cgl* mutant has lost both the capacity to synthesize cysteine and also the capacity to break it down, whereas the *cyuC* mutant has lost the ability to acquire exogenous cystine but has retained the ability to break cysteine down. The *cyuC* mutant would therefore be expected to have serious problems in maintaining high intracellular cysteine

concentrations. This is exactly what we see. The model is consistent with the very high cysteine or cystine concentration requirements for optimal growth of BR11 under aerobic conditions and the evolution of the very high expression level of the CyuC transporter (19).

Unlike *E. coli*, many gram-positive bacteria lack glutathione, but they have other intracellular thiols that perform its function (34). Cysteine is a major intracellular thiol of *Bacillus subtilis* and is important in oxidative defense by S thiolation of thiol-containing proteins (16). In the majority of organisms studied, glutathione is synthesized by the sequential action of γ -glutamylcysteine synthetase and glutathione synthetase, encoded by *gshA* and *gshB*, respectively (21). The genome sequences of *L. reuteri* F275 and 100-23 contain no homolog of *gshB*, but two homologs of *gshA* are present in each. Therefore, the major intracellular thiol of *L. reuteri* is unlikely to be glutathione but may be γ -glutamylcysteine, as has previously been shown to occur in *Leuconostoc* spp. (24). The question as to whether *L. reuteri* is capable of reducing oxidized γ -glutamylcysteine remains open. However, there is a striking similarity between the phenotypes of wild-type BR11 and a glutathione reductase (GshR)-deficient mutant of *Lactobacillus sanfranciscensis* (22). Both have oxygen sensitivity that is relieved by high cysteine concentrations. This shows that a high concentration of exogenous cysteine can substitute for the ability to recycle a cysteine-based redox buffer, such as glutathione. Our current model is that in BR11, intracellular redox recycling of any cysteine-based compound is not efficient and that this explains the very high cysteine concentration requirement in the presence of oxygen. The lack of GshR activity in BR11 (22) and the lack of any *gshR* homologs in the available *L. reuteri* genomes are consistent with this model.

There are at least two ways in which Cgl can synthesize cysteine: by cleaving cystathionine derived from methionine (reverse transsulfuration) and by cleaving cystine to form thio-cysteine (55), which in turn nonenzymatically degrades to cysteine and H₂S (30). Although the cysteine can be further degraded by Cgl, it is not certain whether all of that cysteine will be catabolized. Our results have shown that the reverse transsulfuration pathway in BR11 is not efficient, because methionine is not readily converted to cysteine for oxidative defense, even in the presence of a high concentration of methionine. Despite the inefficiency of the reverse transsulfuration pathway, it appears that the cysteine-forming reaction of Cgl dominates the cysteine degradation reaction, because inactivation of Cgl resulted in growth defects that were relieved by cysteine supplementation. If it were otherwise, one would expect the growth of the *cgl* mutant to be better than that of the wild type because of reduced cysteine degradation.

The CyuC transporter cotranscribed with a Cgl protein is something of a defining feature of *L. reuteri*. Among gram-positive bacteria, only *Lactobacillus casei* and *Lactobacillus rhamnosus* have similar gene arrangements: a *cgl* homolog is located downstream of a *cyuC* homolog, which in turn is downstream of a *cyuA* homolog (D. Barry, unpublished data). The CyuC cystine-binding protein is unusual with respect to solute-binding proteins in gram-positive bacteria in that it is not a lipoprotein and is attached to the cell wall by electrostatic interactions. The consequent potential difficulty in contacting the cell membrane has previously been invoked as a reason for

its expression at near-S-layer levels (45). However, an alternative explanation is that *L. reuteri* is in an evolutionary cul-de-sac as a result of the transcriptional linkage between the CyuC transporter and Cgl. This means that evolutionary pressure to increase CyuC expression will also increase Cgl expression. The low substrate specificity of Cgl allows it to cleave the cystine substrate of the CyuC uptake system and also cysteine that is derived from cystine isomerization. This ultimately wastes the transported cystine with respect to its functioning in oxidative defense. The lack of the ability of methionine to substitute for cysteine in relieving oxidative stress demonstrates that the maximal rate of reverse transsulfuration is low, irrespective of Cgl expression levels. Thus, any increased expression of CyuC will always be compromised by increased Cgl-mediated cystine/cysteine breakdown. This buffering of the effect of increased CyuC expression can potentially lead to the observed very high expression level of the *cgl-cyuC* operon. A phenotypic consequence of this is that BR11 and probably all *L. reuteri* strains are potent producers of H₂S. This is potentially significant because H₂S is toxic and *L. reuteri* is increasingly being consumed in large doses as a probiotic.

Finally, this work demonstrates the profound effect that the routine growth medium used can have on the generally accepted phenotypes of bacterial strains. Based on the typical amino analyses of the protein components of MRS available on the Oxoid website, the concentration of cystine in MRS is approximately 0.7 mM. Our results have shown that MRS contains sufficient cystine to partially but not completely protect BR11 from oxidative stress. As a result, it will grow aerobically, but to a lower OD than when it is grown under anaerobic conditions. Also, it survives poorly when stored aerobically on solid medium (unpublished observations). The tolerance to oxygen is entirely linked to the availability of free cystine or cysteine, with millimolar concentrations being completely oxygen protective.

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