A Peptide Permease Mutant of *Mycobacterium bovis* BCG Resistant to the Toxic Peptides Glutathione and S-Nitrosoglutathione

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Oligopeptides play important roles in bacterial nutrition and signaling. Using sequences from the available genome database for *Mycobacterium tuberculosis* H37Rv, the oligopeptide permease operon (*oppBCDA*) of *Mycobacterium bovis* BCG was cloned from a cosmid library. An *opp* mutant strain was constructed by homologous recombination with an allele of *oppD* interrupted by kanamycin and streptomycin resistance markers. The deletion was complemented with a wild-type copy of the *opp* operon. Two approaches were taken to characterize the peptide transporter defect in this mutant strain. First, growth of wild-type and mutant strains was monitored in media containing a wide variety of peptides as sole source of carbon and/or nitrogen. Among 25 peptides ranging from two to six amino acids in length, none was capable of supporting measurable growth as the sole carbon source in either wild-type or mutant strains. The second approach exploited the resistance of permease mutants to toxic substrates. The tripeptide glutathione (γ -glutamyl-L-cysteinylglycine [GSH]) is toxic to wild-type BCG and was used successfully to characterize peptide uptake in the *opp* mutant. In 2 mM GSH, growth of the wild-type strain is inhibited, whereas the *opp* mutant is resistant to concentrations as high as 10 mM. Similar results were found with the tripeptide S-nitrosoglutathione (GSNO), thought to be a donor of NO in mammalian cells. Using incorporation of [³H]uracil to monitor the effects of GSH and GSNO on macromolecular synthesis in growing cells, it was demonstrated that the *opp* mutant is resistant, whereas the wild type and the mutant complemented with a wild-type copy of the operon are sensitive to both tripeptides. In uptake measurements, incorporation of [³H]GSH is reduced in the mutant compared with wild type and the complemented mutant. Finally, growth of the three strains in the tripeptides suggests that GSH is bacteriostatic, whereas GSNO is bacteriocidal.

Mycobacteria are characterized by long-term survival in the macrophage. Understanding this lifestyle is crucial to answering key questions about mycobacterial pathogenesis. The bacilli infect macrophages early in mycobacterial disease, where they remain protected from specific and nonspecific immune responses and many antibacterial drugs. Yet little is known about intracellular nutrition, i.e., the sources of carbon, nitrogen, and energy and how they are acquired.

Peptides are a valuable form of nutrients, especially for fastidious microorganisms, and in some cases, the growth rate of an amino acid auxotroph can be enhanced with peptides containing the required amino acid (36). Peptide metabolism and transport has been extensively characterized in gram-negative species such as *Escherichia coli* and *Salmonella typhimurium* (13, 27) and in gram-positive organisms (33, 41, 48). In particular, peptide transport and utilization have been well studied in the fastidious organism *Lactococcus lactis* (42).

Peptides often serve as sources of amino acids. Certain auxotrophic strains of *L. lactis* require a functional peptide uptake system for growth on milk protein casein (42, 48). Auxotrophic mutants of *Listeria monocytogenes*, an intracellular pathogen, were examined for growth in cell culture and virulence in mice. In culture, threonine auxotrophs grow poorly on free threonine and quite well on threonine-containing peptides. These auxotrophs showed no difference in growth rate within threonine-

starved J774 macrophages, suggesting that threonine-containing peptides are available for intracytoplasmic growth (26).

Nutrient uptake is crucial in the intracellular survival of bacteria since some nutrients may be severely limited during some stages or compartments of the infection pathway. For example, transport of glutamine was investigated in the intracellular parasite *S. typhimurium*, i.e., a strain with mutations in both synthesis and high-affinity transport of glutamine was attenuated for survival in macrophages, whereas either mutation alone had no effect (18). These results point to the limited accessibility of this amino acid in the intracellular environment. A gene encoding an arginine permease is upregulated during infection of *L. monocytogenes* (17). These experiments suggest that transport plays a crucial role in the control or maintenance of intracellular metabolism.

The most common peptide transporters found among the bacteria are binding protein-dependent permeases. These multicomponent transport systems use directly a high-energy phosphate bond during transport. The actions of up to five proteins contribute to the process: extracytoplasmic binding of the substrate, transfer to one or two membrane-bound permeases for translocation across the cytoplasmic membrane, and ATP hydrolysis by one or two proteins located on the cytoplasmic side of the membrane (47). The energy-requiring step is the hydrolysis of ATP by the ATP-binding subunit: a conformational change is then transmitted to the membrane-bound components that mediate passage through the membrane. The components of these systems are closely related members of the larger structural superfamily called the "ABC (ATP-binding cassette) transporters" (12).

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The genomic sequence of the virulent laboratory strain H37Rv of *Mycobacterium tuberculosis* reveals the presence of two peptide permease operons homologous to dipeptide (*dpp*) and oligopeptide (*opp*) permeases of other organisms (7). In this study, the isolation and characterization of a mutant of BCG lacking a fully functional oligopeptide permease (*opp*) is described. The mutant was constructed by homologous recombination of a copy of a peptide permease gene (*oppD*) interrupted by a selectable marker onto the BCG chromosome.

Analysis of the phenotypes of peptide permease mutants is often difficult due to the presence of more than one permease with overlapping specificities. In addition, the lack of availability of radiolabeled peptides makes kinetic analysis difficult and costly. Toxic peptides are useful tools for the selection and characterization of peptide permease mutants. Here, glutathione (γ -glutamyl-L-cysteinylglycine [GSH]) was shown to be toxic to BCG. GSH and its toxic NO derivative, S-nitrosoglutathione (GSNO), were used to characterize a peptide permease mutant of BCG.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains HB101, DH5 α , and GM48 were used for DNA manipulation and cloning. BCG (Pasteur) was used for all experiments and was subcultured for no more than eight passages in succession. BCG mutant strains were created from early-passage bacteria, expanded to large volumes, and frozen at -70°C to ensure shorter culture duration. Standard DNA manipulations were used (25).

Media, antibiotics, and growth conditions. Middlebrook 7H9 (liquid) and 7H11 (agar) media (Difco) supplemented with glycerol (0.5%) and Tween 80 (0.05%) (Sigma Chemicals) were used for selection and maintenance of BCG ("rich medium"). When used as the sole carbon source, amino acids and oligopeptides were obtained from Sigma. These supplements were included in minimal medium (basal salts) or Sauton's without added L-asparagine at the concentrations given in the legends to the figures (8). Ampicillin was used at a concentration of 50 $\mu\text{g}/\text{ml}$. Kanamycin (Sigma) and streptomycin (Sigma) were used at concentrations of 50 and 100 $\mu\text{g}/\text{ml}$ (respectively) for *E. coli* and at 20 $\mu\text{g}/\text{ml}$ (both) for BCG. GSNO was obtained from Alexis Corporation (San Diego, Calif.) and was >98% pure, as confirmed by thin-layer chromatography (TLC) (acetonitrile-*n*-butanol-toluene-acetic acid-water [1:1:1:1]).

Peptides tested for utilization by BCG. The following peptides were tested as the sole source of carbon and/or nitrogen (all amino acids are L enantiomers unless otherwise indicated): Ala-Ala, Ala-Ala-Ala, Ala-Glu, Ala-His, B-Ala-His, Asp-Asp, Asp-Ala, Asp-Asp-Asp, Asp-Asp-Asp-Asp, Glu-Ala, Ala-Gly-Gly, DL-Ala-DL-Leu-Gly, His-Ala, Leu-Phe, Phe-Val, Val-Phe, Phe-Leu, Leu-Leu, Phe-Phe, Gly-Tyr, Gly-His, Orn-Orn-Orn, Leu-Leu-Leu, and Phe-Phe-Phe. The media used were Basal Salts (8) and Sauton's medium (without amino acids), supplemented with 1/10 the standard amount of A(D)C, omitting the glucose of ADC when the peptide was being tested as the sole carbon source. No background growth was detected in basal medium containing ADC as the carbon and/or nitrogen source.

Construction of BCG deletion strain. Homologous recombination of a cloned and interrupted copy of the *opp* operon was performed. Oligonucleotide primers (forward, ACTCGATGTCTCCATTCAGG; reverse, ATATCGAGTCTGCGTCCAGG) amplifying sequences in *oppC* were used to identify a cosmid containing *opp* sequences from a genomic library of BCG constructed in pYUB18 (14). A 4.5-kb *EcoRI* fragment of DNA encompassing part of the *opp* operon (Rv1280c to Rv1283c) was cloned from a BCG cosmid library in pYUB18 (14) and inserted into pGEM (see Fig. 1). *oppD* was interrupted at the *Clal* site with a 3.4-kb Kan^r-Str^r antibiotic cassette (pSM240, kindly provided by I. Smith) to make pRG6. This plasmid was linearized with *SacI*. The construct afforded 3 kb of direct homology on either side of the antibiotic selection marker. Then, 5 to 10 μg was repeatedly transformed into wild-type BCG (Pasteur) with a typical yield of one to five colonies per microgram of linearized DNA. A total of 45 Kan^r-Str^r colonies were obtained. These candidates were then screened by PCR with oligonucleotide primers flanking the *Clal* site of the Kan-Str resistance marker insertion (forward, TGGGTATCGTCGGCGAATC; reverse, TGCAATGGTTCGGCAATCAG). Of 45 colonies screened, three strains showed amplification products consistent with allele replacement. One of these, BCG(*opp* Δ -19), was further characterized by Southern analyses by using both pRG5 DNA (i.e., a cloned copy of the region used to construct the strain, see Fig. 1) and the Kan-Str marker (data not shown) as probes. The strain BCG(*opp* Δ -19) was used for all subsequent experimentation.

Southern blot analysis. Genomic DNA was prepared from wild-type BCG and BCG(*opp* Δ -19) and cut with *HindIII*. The DNA fragments were separated on a 0.8% agarose gel and probed with the 4.5-kb *EcoRI* fragment (described above) spanning Rv1280c to Rv1283c of the *opp* operon. Since there is a *HindIII*

FIG. 1. Map of *opp* region in *M. tuberculosis* H37Rv and homologous BCG plasmids. (A) Restriction map of *opp* region, derived from Sanger database (7). H, *HindIII*; R, *EcoRI*; C, *Clal*; N, *NheI*. (B) ORFs comprising the *opp* operon and gene assignments (*oppBCDA*). (C) *M. tuberculosis* cosmids from the Sanger database spanning the *opp* operon. pRG5 (uninterrupted subfragment), pRG6 (interrupted fragment), and pRG11 (complementing plasmids) are as described in Materials and Methods.

restriction site in the sequence of the Kan-Str marker used to interrupt the *opp* region (see Fig. 1), the mutant strain DNA exhibits two bands when probed with DNA that flanks the selectable marker's insertion site (see Fig. 2).

Analysis of the toxicity of GSH and GSNO. Mid-log cells (optical density at 600 nm [OD₆₀₀] of 0.4 to 0.6) were diluted to OD₆₀₀ of 0.1 and incubated in 24-well plates in Middlebrook 7H9 plus glycerol, ADC, and 0.05% Tween in the presence or absence of GSH at the concentrations indicated (see Fig. 3). After 3 days, the OD₆₀₀ of the cultures was measured. A second growth assay (see Fig. 5) was performed in 5-ml cultures containing substrates at 2.5 mM. The OD₆₀₀ was measured every 12 h for 4 days.

To measure the effect of GSH, GSNO, or related substrates on [³H]uracil incorporation, 0.5 μCi of [³H]uracil (38.5 Ci/mmol) (New England Nuclear) and peptide were added together to the cells to initiate the experiment. After 16 h, the cultures were precipitated in 10% trichloroacetic acid (TCA) (Sigma) at 4°C for 20 min and then filtered and washed three times with 5 ml of cold 10% TCA over filters (Whatman GF/F, 0.45- μm pore size) prewetted with 10% TCA on a Hoefer 10-place manifold with air vacuum. The filters were dried and transferred to vials with 5 ml of scintillation fluid for determination of radioactivity. Experiments were performed at least three times with each time point in triplicate.

Cytotoxic effects of the peptides were determined by plating dilutions of bacteria on Middlebrook 7H11 agar supplemented with glycerol, ADC, and appropriate antibiotics and counting the CFU.

Incorporation of [³H]GSH. Cells growing in Middlebrook 7H9 (plus glycerol, ADC, and 0.05% Tween) were collected at an OD₆₀₀ of 0.6, washed in basal salts with 0.05% Tween, and concentrated to an OD₆₀₀ of 3.0. The cell suspensions (1 ml) were warmed to 37°C with shaking. The uptake reaction was initiated by the addition of radiolabeled substrate plus unlabeled substrate at a specific activity of 4.48 $\mu\text{Ci}/(\mu\text{mol})$ and to a final concentration of 100 μM . Incorporation was terminated by removal of 0.1-ml samples at the indicated time onto filters (Whatman GF/F, 0.45- μm pore size) prewetted with basal salts. The cells were rinsed quickly (within 10 to 15 s) with three washes of 5 ml of ice-cold basal salts plus Tween 80 on a Hoefer 10-place manifold with air vacuum. Filters with cells thereon were transferred to vials with 5 ml of scintillation fluid for the determination of radioactivity. Counts per minute were normalized to milligrams of protein per 0.1-ml aliquot for each cell suspension; the protein content was determined by Bio-Rad protein assay. Transport assays were performed three times at each time point in triplicate.

RESULTS

Cloning of the *opp* operon of BCG encoding a binding-protein-dependent transporter. A region encoding four genes (Rv1280c to Rv1283c) is contained in the overlapping cosmids MTCY373, MTCY3H3, and MTCY50 of the Sanger database (Fig. 1) (7). The four genes encode homologs of components of an ABC (ATP binding cassette) transporter (12, 13). This class of transporter is comprised of five components, a periplasmic (or extracellular) substrate binding protein, a two-component membrane permease, and two ATP-hydrolyzing subunits. These energy-transducing proteins can be identical or encoded by different genes.

In the *M. tuberculosis opp* operon, the first open reading frame (ORF) in the direction of transcription is Rv1283c, showing 31% identity in a 345-amino-acid overlap with the *E. coli dppB* gene encoding a putative inner membrane permease protein. The second ORF, Rv1282c, is also an inner membrane permease homolog, with 40.7% identity in a 275-amino-acid overlap to *E. coli oppC*. The third ORF, Rv1281c, contains an ATP/GTP-binding site motif and has 48.6% identity in a 319-amino-acid overlap to *dppD* of *Bacillus subtilis*. The last ORF in the operon, Rv1280c, shows 22.1% identity in a 458-amino-acid overlap to the *S. typhimurium oppA* gene, encoding a periplasmic peptide-binding protein homolog. This *M. tuberculosis oppA* gene has a possible N-terminal signal sequence and prokaryotic lipoprotein attachment site. Note that two of the four *opp* genes show the highest homology to equivalent components from the dipeptide operon (*dpp*) of *E. coli* and *B. subtilis*. A 4.5-kb *EcoRI* fragment, including Rv1280c to Rv1282c, was cloned and used to construct a deletion mutant of *oppD* (see Materials and Methods).

The operon is flanked by ORFs directed in the opposite orientation, suggesting that the *opp* promoter regulates the expression of only these four genes. The order of the four genes in this operon is unusual. The majority of ABC transport system operons contain the four or five components in the order A-B-C-D-E-F (4). In *M. tuberculosis* H37Rv, the *oppA* gene is transposed to the terminal position in the operon transcript. Furthermore, the *oppB*, *oppC*, and *oppD* genes are contiguous, whereas there is a 4-bp separation between *oppD* and *oppA*.

Interruption of genomic copy of *opp* in BCG. A 4.5-kb fragment of DNA spanning the *oppC* and *oppA* genes was cloned and interrupted with a Kan-Str selectable marker to construct pRG6 (Fig. 1). The selectable marker was inserted into the *opp* operon to interrupt the expression of *oppD*. The *oppD* gene encodes the two ATP-binding components of the transport system. In some instances, these ATP-binding components are encoded by two genes (*oppD* and *oppF*), but in the case of the *M. tuberculosis* H37Rv *opp* operon there is a single gene encoding this component. Without OppD, the transport system cannot function, since energy supplied by ATP hydrolysis would not be available (12). In addition, polar effects of the marker insertion in *oppD* should interrupt expression of the downstream *oppA* gene, the product of which binds substrate and delivers it to the inner membrane permease proteins encoded by *oppB* and *oppC*.

The plasmid pRG6 (Fig. 1) was used to construct a strain of BCG by homologous recombination. Forty-five Kan^r-Str^r colonies were first screened by PCR (see Materials and Methods). The products of the PCR reactions of 42 of these DNAs were comprised of two bands, one wild type and one increased by the size of the antibiotic marker: these strains were resistant to Kan and Str as a result of single crossover events and are merodiploids. Three strains showed PCR patterns consistent with allele replacement: in each, a single band of the appropriate size was present. One of these, BCG(*oppΔ-19*), was used for all further studies.

The structure of the interrupted allele in BCG(*oppΔ-19*) was further examined by Southern analysis (Fig. 2). Genomic DNA from the wild type and the mutant strain was digested with *HindIII* and probed with a 4.5-kb DNA fragment representing the cloned region. The probe hybridized to a fragment of 21 kb from genomic DNA from wild-type BCG. The probe recognized two bands of 11 and 10 kb from genomic DNA from the mutant strain BCG(*oppΔ-19*). This doublet is due to the presence of a *HindIII* site in the Kan-Str marker used to interrupt the *opp* operon. This, together with the PCR data, indicates

FIG. 2. Southern analysis of genomic DNA from wild-type and (*oppΔ-19*) BCG strains. DNAs from wild-type (lane A) and (*oppΔ-19*) mutant (lane B) cells and plasmid DNAs from pRG5 (lane 1) and pRG6 (lane 2) were digested with *HindIII* and probed with the 4.5-kb fragment representing the internal cloned region from the *opp* operon (see Fig. 1 and Materials and Methods).

that the Kan-Str marker had been recombined onto the chromosome of the BCG(*oppΔ-19*) mutant strain.

A third strain was constructed by using a wild-type copy of the *opp* operon carried on a 11-kb *NheI* fragment. This fragment was inserted into pMV305, an integrating vector that exploits the L5 mycobacteriophage integration system (22). The mutation in the *opp* operon was thus complemented in this strain, BCG(*oppΔ-19*)(*opp*⁺), by a single copy of the wild-type operon. The strain was expected to behave in a manner similar to that of the wild type in the assays described below.

Peptide utilization by wild-type and BCG(*oppΔ-19*). One method of analyzing transport of a substrate is to examine a strain's ability to grow on the substrate as the sole source of carbon and/or nitrogen. Historically, the mycobacteria were thought to be capable of using peptides and proteins for growth (39). Here, 25 di- and tripeptides (see listing and medium composition in Materials and Methods) were tested for utilization as sole carbon and/or sole nitrogen sources by wild-type BCG and BCG(*oppΔ-19*). None of the peptides, when supplied as the sole source of carbon in the medium, was capable of supporting growth of any of these strains. When supplied as the sole source of nitrogen, a small number of peptides from among those tested could support very poor growth. The doubling time of these cultures was in the range of 80 to 100 h. The wild-type and mutant strains showed no significant differences in growth ability on any of these peptides.

Sensitivity of wild type and BCG(*oppΔ-19*) to the toxic effects of GSH and GSNO. A second method of analyzing the phenotype of a peptide transport mutant involves the use of toxic substrates: mutants unable to transport the substrate should be resistant to its toxic effects. The tripeptide GSH is abundant in most eukaryotic and many bacterial cells and is a major protectant against oxidative stress. However, GSH is toxic to BCG. To analyze the effects of GSH on BCG, cultures of wild-type cells growing in Middlebrook 7H9 medium (with glycerol, ADC, and 0.05% Tween) were treated with increasing concentrations of GSH for 3 days. Figure 3 shows that the growth of BCG was inhibited in the presence of 4 mM GSH.

FIG. 3. Inhibition of growth of wild-type and (*oppΔ-19*) BCG by GSH. Exponentially growing cells (OD₆₀₀, 0.4 to 0.6) were diluted to an OD₆₀₀ of 0.1 and incubated in 24-well plates in Middlebrook 7H9 in the presence or absence of GSH at the concentrations indicated. After 3 days, the OD₆₀₀ of the cultures was measured. Black bars, wild-type BCG; hatched bars, BCG(*oppΔ-19*). The experiment is representative of three similar experiments.

The oligopeptide permease mutant BCG(*oppΔ-19*), however, was resistant to the toxic effects of 4 mM GSH. At 8 mM, however, survival of both the mutant and the wild-type cells was affected.

The half-life of GSH at pH 7.0 is approximately 7 h (16). Measurement of growth inhibition after 3 days, therefore, may represent the extent of recovery from growth inhibition and not the level of inhibition itself. To examine the immediate effects of GSH on the growth of wild-type BCG and its *opp* derivative, a metabolic labeling assay was used (6). Log-phase cells were incubated at 37°C with [³H]uracil and peptide at the concentrations indicated for 12 h. Incorporation of [³H]uracil by the cells was measured, and the data are shown in Table 1. GSH at 0.5 mM inhibited [³H]uracil incorporation by more than 50% in wild-type BCG, whereas incorporation by the *oppΔ-19* mutant remained unaffected. Importantly, complementation of the *opp* defect with a wild-type copy of the *opp* operon restored sensitivity to the *oppΔ-19* mutant.

The NO donor, GSNO, is an intermediate in redox signaling (32) and causes oxygen-independent cytostasis in *S. typhimurium* (10). Wild-type BCG is also susceptible to GSNO at 500 μM, and BCG(*oppΔ-19*) is resistant to this concentration as shown in Table 2. When the cells are exposed to 0.5 mM GSNO, there is a 10% reduction in [³H]uracil incorporation by wild-type BCG compared to no measurable reduction of [³H]uracil incorporation in *opp* mutant cells.

TABLE 1. Effect of GSH on [³H]uracil incorporation by BCG strains^a

GSH (mM)	% [³ H]uracil incorporation ± SEM		
	BCG (WT)	BCG(<i>oppΔ-19</i>)	BCG(<i>oppΔ-19</i>)(<i>opp</i> ⁺)
0	100	100	100
0.5	44 ± 5.7	116 ± 8.9	44 ± 11.4
1.0	6 ± 12.7	44 ± 11.5	11 ± 9.7
2.0	0 ± 5.9	50 ± 4.5	0 ± 3.9

^a Data are expressed as the percentage of the level of initial incorporation in counts per minute per 0.1 ml of culture. The extent of [³H]uracil incorporation was measured in triplicate, and the means were normalized to the level incorporated with no added GSH. The experiment shown is representative of three separate experiments. WT, wild type.

TABLE 2. Effect of GSNO on [³H]uracil incorporation by BCG strains^a

GSNO (mM)	% [³ H]uracil incorporation ± SEM		
	BCG (WT)	BCG(<i>oppΔ-19</i>)	BCG(<i>oppΔ-19</i>)(<i>opp</i> ⁺)
0	100	100	100
0.5	89 ± 15.3	132 ± 7.7	92 ± 11.9
1.0	47 ± 5.7	72 ± 13.4	65 ± 8.5
2.0	40 ± 9.5	81 ± 16.2	42 ± 8

^a Data are expressed as the percentage of the level of initial incorporation in counts per minute per 0.1 ml of culture. The extent of [³H]uracil incorporation was measured in triplicate, and the means were normalized to the level incorporated with no added GSNO. The experiment shown is representative of three separate experiments. WT, wild type.

BCG(*oppΔ-19*) differs from its wild type parent only in the recombination of an interrupted genomic copy of the *oppD* gene. Therefore, the basis of the mutant strain's resistance to the toxic effects of GSH and GSNO may be a result of its reduced ability to transport GSH. To examine the uptake of GSH by these BCG strains, incorporation of [³H]GSH by wild-type and mutant cells was measured for 3 h, and the results are shown in Fig. 4. After 3 h, [³H]GSH was incorporated into BCG(*oppΔ-19*) less efficiently than in wild-type BCG. The complemented strain, BCG(*oppΔ-19*)(*opp*⁺), showed levels of [³H]GSH incorporation restored to those of the wild-type strain. Thus, the basis of GSH resistance exhibited by the *opp* mutant is likely the result of reduced uptake of GSH or a derivative thereof.

GSH is cytostatic and GSNO is cytotoxic towards BCG in liquid culture. Wild-type BCG, BCG(*oppΔ-19*), and BCG(*oppΔ-19*)(*opp*⁺) were grown in the presence of GSH and GSNO at 2.5 mM for 48 h, during which period the OD₆₀₀ of the culture increased from 0.1 to 0.6. The growth of the wild type was sensitive to GSH (Fig. 5, closed triangles) during the early stages of the experiment but showed recovery by the end of the

FIG. 4. Uptake of [³H]GSH by wild type, BCG(*oppΔ-19*), and BCG(*oppΔ-19*)(*opp*⁺). Cells growing in Middlebrook 7H9 were collected at an OD₆₀₀ of 0.6, washed in basal salts with 0.05% Tween, and concentrated to an OD₆₀₀ of 3.0. GSH was added at a final concentration of 100 μM, with a specific activity of 4.48 μCi/μmol. Uptake is expressed as counts per minute per milligram of protein. Symbols: ■, wild-type BCG; ▲, BCG(*oppΔ-19*)(*opp*⁺); ○, BCG(*oppΔ-19*). The experiment is representative of three similar experiments.

FIG. 5. Growth of BCG, BCG(*oppΔ-19*), and BCG(*oppΔ-19*)(*opp*⁺) in glutathione and related compounds. Mid-log-phase wild-type BCG (A), BCG(*oppΔ-19*) (B), and BCG(*oppΔ-19*)(*opp*⁺) (C) were washed and resuspended at a starting OD₆₀₀ of 0.1 in complete Middlebrook 7H9 medium plus ADC and Tween 80 containing the following substrates at 2.5 mM: no addition (■), L-Ala-Gly (as a control dipeptide) (◇), GSH (▲), GSNO (●), L-Cys-Gly (□), L-Cys (△), and Gly (○). The OD₆₀₀ was measured every 12 h over 4 days. The experiment is representative of four similar experiments.

experiment. This is most likely due to the short half-life of GSH in solution (37). As expected, BCG(*oppΔ-19*) was resistant to the toxic effects of GSH. The complemented mutant strain, however, consistently showed resistance to GSH during growth, in contrast to the sensitivity shown in the metabolic assay described above. Overall, these growth patterns suggest that GSH has a cytostatic effect on wild-type BCG.

All three strains did not grow in the presence of GSNO (Fig. 5, closed circles). GSNO also has a short half-life in aqueous solution (32). The growth inhibition of all three BCG strains by GSNO suggests that GSNO acted early against the cells and is cytotoxic. This was confirmed by removing cells at various time points along the growth curve and plating the dilutions in the absence of GSNO or GSH. The GSNO-treated cultures showed no survival on Middlebrook 7H9 plates (data not shown). However, the numbers of CFU representing all three strains from GSH-treated cultures were proportional to the ODs measured in the cultures (data not shown). Although the BCG *opp* mutant shows resistance to similar levels of GSH and GSNO when evaluated by [³H]uracil incorporation, we conclude from these experiments that GSNO, but not GSH, is cytotoxic against wild-type BCG.

Peptide and amino acid components of GSH are not toxic to BCG. The basis of GSH toxicity against BCG is not known. In

S. typhimurium, GSNO itself is not transported into the cell. A periplasmic transpeptidase encoded by the *ggt* gene removes the γ -glutamyl moiety. The dipeptide *S*-nitroso-L-cysteinylglycine is then thought to enter the cell by the dipeptide permease (*dpp*) system (10). To determine whether the dipeptide L-Cys-Gly or its constituent amino acids were responsible for toxicity against BCG, these components were tested in both metabolic and growth assays. None of the components of the tripeptide showed toxicity against BCG during growth (Fig. 5). However, Table 3 shows that treatment of wild-type and complemented mutant cells with the dipeptide L-Cys-Gly shows some inhibition of [³H]uracil incorporation; this inhibition was not evident in BCG(*oppΔ-19*) cells. L-Cys alone showed equivalent but modest toxicity against all three strains (Table 4). Finally, Gly had no effect on [³H]uracil incorporation by any of the three strains (Table 5).

DISCUSSION

A mutant strain of BCG was constructed in which the *oppD* gene was interrupted with a selectable marker. Sequence homology indicates that *oppD* encodes the ATPase component of a binding protein-dependent transport system. This component is positioned as a dimer of peripheral membrane proteins

TABLE 3. Effect of L-Cys-Gly on [³H]uracil incorporation by BCG strains^a

L-Cys-Gly (mM)	% [³ H]uracil incorporation $\pm$ SEM		
	BCG (WT)	BCG(<i>oppΔ-19</i>)	BCG(<i>oppΔ-19</i>)(<i>opp</i> ⁺)
0	100	100	100
0.5	106 $\pm$ 9.8	95 $\pm$ 6.7	85 $\pm$ 11.3
1.0	61 $\pm$ 7.9	91 $\pm$ 4.7	74 $\pm$ 11.5
2.0	70 $\pm$ 4.5	111 $\pm$ 9.3	76 $\pm$ 12.3

^a Data are expressed as the percentage of the level of initial incorporation in counts per minute per 0.1 ml of culture. The extent of [³H]uracil incorporation was measured in triplicate, and the means were normalized to the level incorporated with no added L-Cys-Gly. The experiment shown is representative of three separate experiments. WT, wild type.

TABLE 4. Effect of L-Cys on [³H]uracil incorporation by BCG strains^a

L-Cys (mM)	% [³ H]uracil incorporation $\pm$ SEM		
	BCG (WT)	BCG(<i>oppΔ-19</i>)	BCG(<i>oppΔ-19</i>)(<i>opp</i> ⁺)
0	100	100	100
0.5	89 $\pm$ 15.7	76 $\pm$ 8.4	85 $\pm$ 14.1
1.0	104 $\pm$ 21.3	85 $\pm$ 14.9	75 $\pm$ 6.6
2.0	84 $\pm$ 12.8	76 $\pm$ 8.2	71 $\pm$ 11.1

^a Data are expressed as the percentage of the level of initial incorporation in counts per minute per 0.1 ml of culture. The extent of [³H]uracil incorporation was measured in triplicate, and the means were normalized to the level incorporated with no added L-Cys. The experiment shown is representative of three separate experiments. WT, wild type.

TABLE 5. Effect of Gly on [³H]uracil incorporation by BCG strains^a

Gly (mM)	% [³ H]uracil incorporation ± SEM		
	BCG (WT)	BCG(<i>oppΔ-19</i>)	BCG(<i>oppΔ-19</i>)(<i>opp</i> ⁺)
0	100	100	100
0.5	123 ± 16.2	147 ± 7.2	18 ± 5.6
1.0	111 ± 18.3	101 ± 7.7	87 ± 8.7
2.0	121 ± 23.2	94 ± 10.0	83 ± 13.5

^a Data are expressed as the percentage of the level of initial incorporation in counts per minute per 0.1 ml of culture. The extent of [³H]uracil incorporation was measured in triplicate, and the means were normalized to the level incorporated with no added Gly. The experiment shown is representative of three separate experiments. WT, wild type.

localized on the cytoplasmic side of the membrane. These proteins contribute no substrate specificity to the transport system; rather, they function to couple ATP hydrolysis to translocation of substrate across the membrane. Downstream of this ORF, separated by 4 bp, is the *oppA* homolog, the substrate binding protein. The number of binding proteins in ABC transport systems usually exceeds that of the membrane components, at ratios of as high as 30- to 50-fold, as is the case with the maltose- and histidine-binding proteins (4). The alterations in peptide uptake exhibited by the BCG *opp* mutant suggest that the *oppD* insertion exerts a polar effect on *oppA* gene expression as well, although this remains to be confirmed by, for example, immunodetection.

Two approaches to phenotypic characterization of the BCG(*oppΔ*) mutant were taken. Utilization of peptides as sole source of carbon or nitrogen can be compared between the wild-type and mutant strains. *Mycobacterium smegmatis* can use a wide range of peptides to support growth (2). But wild-type BCG was unable to grow on 25 peptides supplied as the sole carbon source, and extremely poor growth was observed on a small number of peptides supplied as the sole nitrogen source. The inability of BCG to grow on peptides as the sole carbon or nitrogen source may be due to insufficient uptake. Transport of several amino acids has been shown to be limiting for growth in *E. coli* and *Klebsiella* spp., and mutations resulting in increased transport of the substrate in question can overcome the negative growth phenotype (28). Whether the inability of BCG to utilize peptides for growth is due to insufficient transport can be tested by overexpression of the *opp* system.

A fruitful approach to the characterization of nutrient transport mutants is to examine resistance to toxic substrate analogs. Two toxic peptides commonly used with bacteria are triornithine, to which mycobacteria are entirely insensitive (N. D. Connell, unpublished data), and bialaphos (L-alanylalanylphosphothricin). Bialaphos is a tripeptide comprised of two alanine residues and a phosphothricin moiety. After transport by the *opp* system, bialaphos is cleaved by intracellular peptidases. The phosphothricin moiety is a glutamate analog that binds irreversibly to glutamine synthetase and kills the cell. *opp* mutants of *B. subtilis* (43) and *Streptomyces coelicolor* (34) are resistant to bialaphos. Mycobacteria are sensitive to bialaphos, and the *opp* mutant described here is fully sensitive to the drug (A. Bhatt and N. D. Connell, unpublished data). A likely interpretation of this result is that the BCG *Opp* characterized in this study does not transport the tripeptide bialaphos.

The tripeptide GSH, found in most living cells, was used here to characterize the BCG(*oppΔ-19*) mutant. A range of functions has been attributed to the peptide, including cofactor function, acting as a transporter component, providing an al-

ternative source of sulfur, and participation in cellular processes such as DNA and protein synthesis, regulation of enzyme activity, and membrane function. In bacteria, GSH is found in facultative and aerobic bacteria but not in strict anaerobes (37).

We have shown that, surprisingly, GSH is toxic to mycobacteria. Mycobacteria and other actinomycetes do not synthesize GSH. Rather, they produce mycothiol [1-D-myo-inosityl-2-(N-acetyl-L-cysteinyl)amido-2-deoxy-α-D-glucopyranoside; MSH] in millimolar amounts (1). MSH has been isolated from a number of mycobacterial species, including *Mycobacterium smegmatis*, *Mycobacterium bovis*, and *M. tuberculosis* H37Rv (1, 5, 31, 45).

The basis of the toxicity of GSH to mycobacteria is unknown and not previously reported. One possibility is that the presence of high concentrations of GSH may result in an imbalance in a bacterium containing an alternative thiol for regulating reduction/oxidation activity (i.e., mycothiol).

Interestingly, GSH is similar in structure to penicillin precursors produced by *Penicillium* and *Cephalosporium* spp., and the β-lactam form of GSH is penicillin-like. Spallholz has hypothesized that GSH is an evolutionary precursor of antibiotics produced by higher eukaryotes before the emergence of cellular immunity (44). Mycobacteria may possess some intrinsic sensitivity to this structure.

Nitric oxide (i.e., NO) and related reactive nitrogen intermediates are thought to be major antimicrobial agents produced during the host defense response (23, 24, 29, 30). In view of the high levels (millimolar concentrations) of GSH found in mammalian cells, the nitrosothiol GSNO is a strong candidate for an in vivo NO donor. In a genetic screen for *Salmonella* mutants resistant to GSNO, De Groote et al. recovered mutants with defects in *dppD* and *dppA* function (10), homologs of the two genes interrupted in the BCG mutant described here.

We have shown that GSNO is cytotoxic for wild-type BCG at concentrations similar to those to which *Salmonella* is sensitive (1 to 2 mM). While the data demonstrate reduced uptake of both GSH and GSNO by the *opp* BCG mutant, the S-nitrosothiol clearly kills both mutant and wild-type cells in culture. GSH, on the other hand, is toxic only to the wild-type strain. It is possible that GSNO, but not GSH, is transported by more than one permease. Alternatively, unlike *Salmonella*, BCG may be sensitive to extracellular NO provided by GSNO, and transport of the nitrosothiol, either as a tri- or dipeptide, is not required for toxicity.

Note that the two methods of analysis of toxicity used ([³H]uracil incorporation, Tables 1 to 5, and growth, Fig. 5) yielded some inconsistencies in resistance levels. These are likely the result of differences between the two assays. [³H]uracil incorporation is a general evaluation of the metabolic state of the cells, since [³H]uracil is incorporated into metabolic pools, entering first RNA and later other macromolecules by degradation and reincorporation. The growth curves are a less sensitive indicator of the effects of these peptides on metabolism.

Uptake of GSNO in *Salmonella* and *E. coli* is dependent on the *ggt* locus, encoding the γ-glutamyltranspeptidase (γGT) (46). γGT transfers a γ-Glu group to an acceptor amino acid or peptide, releasing the dipeptide S-nitrosocysteinylglycine. This points to the possibility that the NO dipeptide is the actual toxic species in the toxicity of GSNO against *Salmonella*. γGT activity has been described in a number of species of mycobacteria (*M. smegmatis* and *Mycobacterium avium*) (20, 40), and two ORFs homologous to the *ggt* gene are present in the Sanger database (Rv0773c and Rv2394). The latter contains a clear lipoprotein motif, suggesting that a transpeptidase is localized at the cell surface in mycobacteria (7).

The data in Tables 3, 4, and 5 suggest that the dipeptide L-Cys-Gly may be at least partially responsible for the toxicity of GSH, since we have demonstrated a low-level toxicity of L-Cys-Gly against BCG. The dipeptide is not active against the *opp* mutant. Further, the data in Table 4 suggests that L-Cys may be a toxic component of both the tri- and dipeptides. Since this amino acid enters cells by an as-yet-unidentified amino acid permease, all three strains are sensitive to its low-level toxicity. Finally, Gly has no effect on any of the three strains.

Several reports point to a crucial role of peptide transporters in microbial cell signaling and virulence. For example, di- and oligopeptide permeases were identified among a number of mutant loci affecting growth and survival of *Staphylococcus aureus* in multiple infection environments (9). In *Borrelia* spp., peptide-binding proteins of ABC-type transporters were found to be conserved those species causing Lyme disease (19). In the group A streptococci, the expression of the cysteine protease is reduced in a dipeptide permease mutant, and expression of the *dpp* operon is under the control of the Mga virulence regulator (38). The survival of the *opp* mutant of BCG in cultured, unactivated murine macrophages was unimpaired compared with its wild-type parent (data not shown). This result suggests that, in this assay, access to small peptides is not a major nutritional requirement of BCG.

In addition to the *opp* operon, the *M. tuberculosis* database contains an operon (Rv3663c to Rv3666c) homologous to the *dpp* operon of *E. coli* and *B. subtilis*. A tripeptide permease system (*tpg*) is present in enteric bacteria (3, 4) and *L. lacti* (11); a *tpg* operon has not been identified in the *M. tuberculosis* genome. The specificity of peptide transport systems has been established by transport studies combining single and multiple mutants, peptides, and their analogs (27, 36). The *Opp* of enteric bacteria transports any peptide up to six amino acids (13, 35), whereas the *Opp* of *L. lactis* is restricted to four to eight amino acids (21). The *Dpp* of enteric bacteria is specific for dipeptides, and the *Tpp* is specific for hydrophobic tripeptides (15). The data presented in this study suggest that the *Opp* of BCG may resemble that of *E. coli* and *Salmonella*, transporting both di- and tripeptides.

An alternative interpretation is that the *opp* designation assigned to this particular operon in the *M. tuberculosis* H37Rv database is in error and that the operon described here, spanning ORFs Rv1280c to Rv1283c, actually encodes a dipeptide permease system. This possibility is suggested by four observations. First, mutations in *dpp* confer resistance to GSNO in *Salmonella* spp. (10). Second, as mentioned earlier, two of the four genes in the *opp* operon of *M. tuberculosis* (Rv1283c and Rv1281c) show higher homology to *dpp* components of other species than to *opp* components (7). Third, unlike *opp* mutants of *Bacillus* (43) and *Streptomyces* (34), the BCG *opp* mutant described here is not resistant to the toxic tripeptide bialaphos. Fourth, the BCG *opp* mutant is resistant to the toxic effects of both GSH and L-Cys-Gly.

Further studies are required to understand peptide discrimination of the mycobacterial permeases. To this end, our laboratory has cloned the second annotated peptide permease (*dpp*) (Rv3663c to Rv3666c) from BCG and is engaged in constructing two additional mutants (*dpp* Δ and a double *dpp* Δ *opp* Δ mutant) to complement studies with the *opp* Δ mutant described here. Finally, construction of such mutants in *M. tuberculosis*, currently under way, will enable the analysis of the role of peptide transport and metabolism in mycobacterial pathogenesis.

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