JOURNAL OF BACTERIOLOGY, June 2005, p. 3839–3847 Vol. 187, No. 11
0021-9193/05/$08.00+0 doi:10.1128/JB.187.11.3839–3847.2005

A Multidomain Fusion Protein in *Listeria monocytogenes* Catalyzes the Two Primary Activities for Glutathione Biosynthesis

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Received 16 January 2005/Accepted 24 February 2005

Glutathione is the predominant low-molecular-weight peptide thiol present in living organisms and plays a key role in protecting cells against oxygen toxicity. Until now, glutathione synthesis was thought to occur solely through the consecutive action of two physically separate enzymes, γ-glutamylcysteine ligase and glutathione synthetase. In this report we demonstrate that *Listeria monocytogenes* contains a novel multidomain protein (termed GshF) that carries out complete synthesis of glutathione. Evidence for this comes from experiments which showed that in vitro recombinant GshF directs the formation of glutathione from its constituent amino acids and the in vivo effect of a mutation in GshF that abolishes glutathione synthesis, results in accumulation of the intermediate γ-glutamylcysteine, and causes hypersensitivity to oxidative agents. We identified GshF orthologs, consisting of a γ-glutamylcysteine ligase (GshA) domain fused to an ATP-grasp domain, in 20 gram-positive and gram-negative bacteria. Remarkably, 95% of these bacteria are mammalian pathogens. A plausible origin for GshF-dependent glutathione biosynthesis in these bacteria was the recruitment by a GshA ancestor gene of an ATP-grasp gene and the subsequent spread of the fusion gene between mammalian hosts, most likely by horizontal gene transfer.

Glutathione (γ-glutamyl-cysteinyl-glycine) (GSH) is the predominant low-molecular-weight peptide thiol present in living organisms. In bacteria it plays a pivotal role in many metabolic processes, chief among which are thiol redox homeostasis, protection against reactive oxygen species, protein folding, and provision of electrons via NADPH to reductive enzymes, such as ribonucleotide reductase. Low-molecular-weight nonribosomal peptides are assembled by the action of versatile multimodular enzymes termed nonribosomal peptide synthetases (NRPS) (22, 37) or through the consecutive actions of individual enzymes. GSH synthesis is a prime example of the latter, and GSH is made in a highly conserved two-step ATP-dependent process by two unrelated peptide bond-forming enzymes (21). The γ-carboxyl group of L-glutamate and the amino group of L-cysteine are ligated by the enzyme γ-glutamylcysteine ligase (encoded by *gshA*) to give γ-glutamylcysteine, which is then condensed with glycine in a reaction catalyzed by glutathione synthetase (encoded by *gshB*) to form GSH. Most gram-positive bacteria do not contain GSH (9). However, a broad survey of the distribution of thiols in microorganisms revealed that several species of gram-positive bacteria, including *Listeria*, streptococci, and enterococci, produce significant amounts of GSH (23). The source of GSH in these bacteria has remained a puzzle, since their genomes do not contain a canonical *gshB* gene. The recent paper of Copley and Dhillon provides a clue to the origin of this GSH (7). These authors identified in the genomes of *Listeria monocytogenes*, *Listeria innocua*, *Clostridium perfringens*, and *Pasteurella multocida* an open reading frame (ORF) that is predicted to contain an N-terminal domain that encodes a molecule significantly related to bacterial γ-glutamylcysteine ligases (GshA) and a C-terminal domain that encodes a molecule that bears little resemblance to typical bacterial glutathione synthetases (GshB) but is clearly related to the ATP-grasp superfamily of proteins (11). All of these enzymes carry out ATP-dependent formation of peptide bonds between a carboxylate group of one substrate and an amino, imino, or thiol group of another substrate, and their catalytic mechanisms are likely to include formation of acylphosphate intermediates (11, 41). Despite their low sequence similarity and the different chemical reactions that they catalyze, the crystal structures of these enzymes reveal considerable structural similarity (38). Here, we report the presence in *L. monocytogenes* of a multidomain fusion protein, Lmo2770, that integrates the two catalytic activities and defines a new route for glutathione synthesis. Recombinant Lmo2770 directs formation of glutathione in vitro. We also show that the *lmo2770* gene has orthologs in otherwise distantly related bacteria, most of which are mammalian pathogens. Below we refer to the listerial fusion protein as GshF and the gene encoding it as *gshF*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *L. monocytogenes* wild-type strain EGD-e (= ATCC BAA-679), the isogenic mutant GshF466, and a revertant of GshF466 were cultured at 37°C in brain heart infusion medium (BHI) (Gibco) for routine growth studies and in tryptic soy broth (TSB) (Sigma Chemicals) supplemented with 25 mM glucose for thiol analyses. The temperature-sensitive shuttle vector pG$^+$host4 (4) was used for construction of insertion mutants and carries an erythromycin resistance gene. *Escherichia coli* TG1 served

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as the intermediate host for cloning (4) and was propagated at 37°C in Luria-Bertani broth.

Construction of the *L. monocytogenes* mutant GshF466 and of the revertant. A 410-bp internal fragment of the *L. monocytogenes lmo2770* gene (*gshF*) spanning nucleotides 991 to 1401 was amplified by PCR using oligonucleotide primers GSHU (forward primer; 5-′AATCAAAGGATCCCTTTCAGAAGATCG-3′), creating a BamHI site (underlined), and GSHD (reverse primer; 5′-AATAGC GAATTCCATATGATTTGGGTG-3′), creating an EcoRI site (underlined), and cloned into the pG$^+$host4 shuttle vector to form pSGhs1. The recombinant pSGhs1 plasmid was introduced by electroporation into *L. monocytogenes* EGD-e, and transformants were selected at 30°C on BHI agar supplemented with 5 μg/ml erythromycin. A positive clone was grown at 30°C in BHI containing 5 μg/ml erythromycin and spread on BHI agar plates containing 5 μg/ml erythromycin, and the plates were incubated for 2 days at 42°C (the nonpermissive temperature for pG$^+$host4 replication). Plasmid integration via homologous recombination yielding the GshF466 disruptant mutant strain was verified by PCR. Proper integration was verified by sequencing across the junction sites of the vector and inserted fragment. The GshF466 revertant was obtained after infection of J774 mouse macrophage-like cells (see below) and was initially identified by colony size (the GshF466 mutant formed small colonies on BHI or TSB agar) and loss of the plasmid-encoded erythromycin resistance. Loss of the plasmid insertion was confirmed by PCR.

Infection assays for survival and multiplication of *L. monocytogenes* EGD-e and the GshF466 mutant in J774 mouse macrophage-like cells and Caco-2 enterocyte-like cells. J-774A.1 (= DSMZ ACC 170) or Caco-2 (= DSMZ ACC 169) cells were seeded into 12-well cell culture plates (Greiner-Bio One, Germany) and grown to confluence in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum. Cultures were infected with *L. monocytogenes* EGD-e and GshF466, and bacterial survival and intracellular multiplication were determined essentially as described previously (10), with minor modifications as described by Karunasagar et al. (14). A multiplicity of infection of one bacterium per cell was used for J774 cells, and a multiplicity of infection of 10 bacteria per cell was used for Caco-2 cells. At 30 min and 1 h after infection, aliquots of cells were washed twice with phosphate-saline buffer to remove adherent bacteria, and the infected cells were lysed by addition of 1 ml of distilled water containing 0.1% Triton X-100. Numbers of viable bacteria, expressed in CFU, were determined from serial dilutions on BHI agar. Gentamicin (10 μg/ml) was added to the remaining cells to kill extracellular bacteria, and at 3, 5, and 7 h postinfection intracellular bacteria were recovered and enumerated as described above.

Microscopic visualization of infection of *L. monocytogenes* EGD-e and the GshF466 mutant in Caco-2 enterocyte-like cells. *L. monocytogenes* EGD-e and GshF466 were transformed with plasmid pLSV16-P*actA-gfp.* This plasmid enabled expression of the *gfp* gene (encoding the green fluorescent protein) under the control of the *actA* promoter but only after escape of the bacterium into the cytosol of the infected host cells (5). Caco-2 enterocyte-like cells were infected as described above. At 6 h and 24 h postinfection, the infected cell monolayers were inspected by phase-contrast and fluorescence microscopy with a DMIIRB inverted microscope (Leica, Germany). The phase-contrast and fluorescence micrographs obtained were overlaid electronically.

Assay for sensitivity to hydrogen peroxide, diamide, and τ-butyl hydroperoxide. *L. monocytogenes* EGD-e and GshF466 and the GshF466 revertant were grown at 37°C in BHI to the mid-log phase, and 0.1-ml portions of the cultures were spread onto BHI agar plates. Filter paper disks (diameter, 5 mm) were placed on the plates and soaked with different amounts and concentrations of hydrogen peroxide, diamide, and τ-butyl hydroperoxide (see Table 3). The plates were incubated at 37°C overnight, and the diameters of the zones of inhibition of bacterial growth were measured.

Preparation of cell extracts for high-performance liquid chromatography (HPLC) analysis of thiols. Cell extracts were analyzed for low-molecular-weight thiols as their bimane derivatives as previously described (23, 24). Quantification of GSH and γ-glutamylcysteine was performed by measurement of the peak areas in chromatogram profiles and comparison with known thiol standards run in parallel.

Construction of the GshF expression vector. To construct pET28a(+)::*gshF*, the complete *lmo2770* sequence was amplified by PCR using forward primer 5′-GAGCTCATGATAAAACTTGATATGAAC-3′ and reverse primer 5′-GA GAGAGCTCCCGTCAAATAAGAAATCTAAAATC-3′. The amplified fragment was eluted from a 1% agarose gel using a QIAGEN QIAquick gel extraction kit, ligated to the Promega pGEM T-Easy vector, and electroporated into *E. coli* XL1-blue. Positive transformants were detected by blue-white screening and by colony PCR, and the DNA insert was sequenced to verify its integrity. pGEM::*gshF* was digested with restriction endonuclease BspHI (New England Biolabs), electrophoresed in 1% agarose, eluted, and digested with restriction endonuclease SacI (Fermentas). The expression vector pET28a(+) (Novagen) was digested with restriction endonuclease NcoI (Fermentas), electrophoresed in 1% agarose, eluted, and digested with the SacI restriction endonuclease (Fermentas). T4 DNA ligase (GibcoBRL) was used to ligate the two SacI DNA fragments to obtain pET28a(+)::*gshF*, which was electroporated into XL1-blue cells, and transformants were selected for kanamycin resistance. The DNA insert in pET28a(+)::*gshF* and the adjacent DNA regions were sequenced to verify the correctness of the construct. Plasmid DNA was prepared using a QIAGEN midi kit and a Roche High Pure plasmid isolation kit. The pET28a(+)::*gshF* construct expresses GshF containing a six-His tag at its C terminus.

Protein overexpression. Overnight cultures of *E. coli* BL-21 λ(DE3) harboring pET28a(+)::*gshF* were diluted to 0.1% (vol/vol) in Luria-Bertani broth containing kanamycin and chloramphenicol (50 μg/ml and 30 μg/ml, respectively) and grown aerobically at 37°C. At an absorbance at 600 nm of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added to a concentration of 1 mM. The cells were incubated for 5 h at 25°C to obtain an optical density of 3.7 and were harvested by centrifugation at 4,000 × *g* for 20 min at 4°C. The supernatant was discarded, and the cell pellet was stored at −20°C.

Protein purification. Frozen cells were thawed and suspended in 50 mM Tris-HCl (pH 8.1), 1 g of wet cell pellet per 4 ml buffer. EDTA-free protease inhibitor cocktail (Roche Complete Mini) was added, and the cell suspension was sonicated. RNase A (10 μg/ml) and DNase I (5 μg/ml) were added (Sigma), and the mixture was centrifuged at 4,000 × *g* for 20 min at 4°C. The supernatant was equilibrated against 50 mM Tris-HCl (pH.8), 300 mM NaCl, 5 mM imidazole, loaded on a high-capacity Ni^{2+}-CAM resin column (Sigma), and washed with the loading buffer. Stepwise elution was performed with 5 mM, 10 mM, 50 mM, 100 mM, and 250 mM imidazole with fixed buffer and salt concentrations. Recovery of recombinant protein was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 9% acrylamide gel.

γ-Glutamylcysteine ligase and glutathione synthetase activity assays. γ-Glutamylcysteine ligase and glutathione synthetase activities were determined on the basis of ADP formation using a pyruvate kinase-lactate dehydrogenase coupled assay (29).

Sequence analyses. GshF amino acid residues 6 to 355, corresponding to the glutamylcysteine ligase domain (PF04262) of bacterial glutamylcysteine ligases, and amino acids 516 to 761, corresponding to the ATP-grasp domain, were used as the queries to retrieve homologs of these domains from databases. Database searches were carried out by using PSI- and PHI-BLAST (3) and the nonredundant protein database at the National Center for Biotechnology Information. Identification and analysis of domain architectures were performed with the SMART (Simple Modular Architecture Research Tool) program (17, 28). Sequence alignment was performed with Clustal W (http://www.ebi.ac.uk/clustalw/index.html) (34) using the default parameters. Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 3.0 (16). The neighbor-joining and minimal-evolution methods, based on the distance matrix calculated for all pairs from the sequence alignment, were used for tree reconstruction. The confidence limits of branch points were estimated from 1,000 bootstrap replicates. The *L. monocytogenes* genome data were obtained from a website (http://genolist.pasteur.fr/ListiList). The EMBL/GenBank accession number for the *L. monocytogenes* EGD-e genome sequence is AL591824. Table 1 lists the bacterial strains, protein sequences, and databases used in this study for preparation of sequence alignments and phylogenetic trees.

RESULTS AND DISCUSSION

GshF is a fusion protein consisting of γ-glutamylcysteine ligase and ATP-grasp domains. Figure 1A shows the domain structure of the putative *L. monocytogenes* 776-amino-acid GshF (Lmo2770 [12]). The N-terminal portion of the protein, comprising amino acids 1 to 441, exhibits moderate sequence identity with the 518-amino-acid *E. coli* GshA protein (24% identity for the sequences; *E* value, 4e-44) and lacks the ~50-amino-acid segment present in the GshA C terminus; residues 6 to 355 form the glutamylcysteine ligase domain (PF04262) of bacterial glutamylcysteine ligases (EC 6.3.2.2). The C-terminal portion of the putative protein, comprising amino acids 450 to 776, contains the ATP-grasp domain consisting of residues 516 to 761. Employing the BLAST2seq, Lalign, or SSEARCH program (using the Smith-Waterman algorithm [version 2.0u4,

TABLE 1. Bacterial strains, protein sequences, and databases used in this study

Organism	Annotation[a]	Locus name	Database[b]
Actinobacillus pleuropneumoniae serovar 1 strain 4074	GshF	Aple02002114	OUACGT
Actinobacillus actinomycetemcomitans HK1651	GshF		OUACGT
Clostridium perfringens 13	GshF	Cpe1573	
Desulfotalea psychrophila LSv54	GshF	DP1233	
Enterococcus faecalis V583	GshF	EF3089	
Enterococcus faecium DO	GshF	Efae03001459	Joint Genome Institute
Haemophilus somnus 129PT	GshF	Hsom02000231	Joint Genome Institute
Listeria monocytogenes EGD-e	GshF	LMO2770	
Listeria innocua Clip11262	GshF	LIN2913	
Mannheimia succiniciproducens MBEL55E	GshF	MS1683	
Pasteurella multocida Pm70	GshF	PM1048	
Streptococcus agalactiae NEM316	GshF	GBS1862	
Streptococcus mutans UA159	GshF	SMU.267c	
Streptococcus suis P1/7	GshF		Sanger Centre
Streptococcus gordonii Challis NCTC7868	GshF		TIGR
Streptococcus sanguinis SK36	GshF		Virginia Commonwealth University
Streptococcus sobrinus 6715	GshF		TIGR
Streptococcus thermophilus CNRZ1066	GshF	STR1413	
Streptococcus uberis 0140J	GshF		Sanger Centre
Lactobacillus plantarum WCFS1	GshF	LP_2336	
Lactobacillus plantarum WCFS1	GshA	LP_2324	
Clostridium acetobutylicum ATCC 824	GshA	CAC1539	
Clostridium acetobutylicum ATCC 824	ATP-grasp	CAC1540	
Bordetella parapertussis 12822	GshA	BPP4089	
Leptospira interrogans serovar Lai 56601	GshA	LA2106	
Leptospira interrogans serovar Lai 56601	ATP-grasp	LA2107	
Bordetella bronchiseptica RB50	GshA	BB4560	
Acinetobacter sp. strain ADP1	GshB	ACIAD3518	
Buchnera aphidicola Bp	GshB	BBP490	
Burkholderia mallei ATCC 23344	GshB	BMA3214	
Escherichia coli K-12 strain MG1655	GshB	B2947	
Pseudomonas aeruginosa PAO1	GshB	PA0407	
Shewanella oneidensis MR-1	GshB	SO0831	
Vibrio cholerae O1 biovar Eltor strain N16961	GshB	VC0468	
Bordetella bronchiseptica RB50	GshB	BB2152	
Bordetella pertussis Tohama I	GshB	BP1499	
Acinetobacter sp. strain ADP1	MurC	ACIAD1279	
Anabaena (*Nostoc*) sp. strain PCC7120	CphA	ALL3879	
Bordetella bronchiseptica RB50	CphA	BB3584	
Clostridium perfringens 13	CphA	CPE2213	
Gloeobacter violaceus PCC 7421	CphA	GVIP562	
Nitrosomonas europaea ATCC 19718	CphA	NE0923	
Escherichia coli K-12 strain MG1655	DdlB	B0092	
Pasteurella multocida Pm70	DdlB	PM0144	
Pseudomonas aeruginosa PAO1	DdlB	PA4410	

[a] Annotation of proteins in databases: the GshF annotation is from this study.
[b] Information was obtained from the GenBank database unless indicated otherwise. TIGR, The Institute for Genome Research. OUACGT, Oklahoma Advanced Center for Genome Technology.

April 1996]) (26, 31), we could not detect any significant similarity between the C-terminal portion of GshF and *E. coli* GshB. The ~160-amino-acid segment connecting the N and C domains of GshF exhibits high sequence identity with the corresponding regions in related ORFs from *L. innocua*, *C. perfringens*, and *P. multocida*, as well as some other bacteria (more than 40% identity between the sequences; *E* values, 1e-11 and lower [see below]). Figure 1B shows a putative gene fusion event that occurs in the formation of GshF-like proteins, as determined by using FusionDB (http://igs-server.cnrs-mrs.fr/FusionDB/), a database of bacterial and archaeal gene fusion events (8, 32).

GshF is a multimodular protein that carries out complete synthesis of glutathione. To determine if the *L. monocytogenes* GshF protein is responsible for synthesis of GSH in vivo, *L. monocytogenes* strain EGD-e (wild type) and mutant GshF466 (containing plasmid pSGhs1 inserted into *lmo2770* at the amino acid 466 position) were grown in TSB, a glutathione-free medium, and GSH was assayed in cell extracts after thiols were derivatized with the thiol-specific reagent monobromobimane, followed by separation by HPLC (24). Figures 2A and B show that disruption of the C-terminal ATP-grasp domain in GshF466 abolished GSH synthesis and led to accumulation of the intermediate γ-glutamylcysteine. A revertant of GshF466, in which the plasmid insert had spontaneously excised, ostensibly resulting in an intact *gshF* gene, exhibited wild-type levels of GSH (Fig. 2C), demonstrating that the plasmid insertion alone and not secondary mutations were responsible for the phenotype observed.

We next tested whether the GshF fusion protein is able to direct formation of GSH in an in vitro system. Purified recombinant GshF was incubated with L-glutamate, L-cysteine, gly-

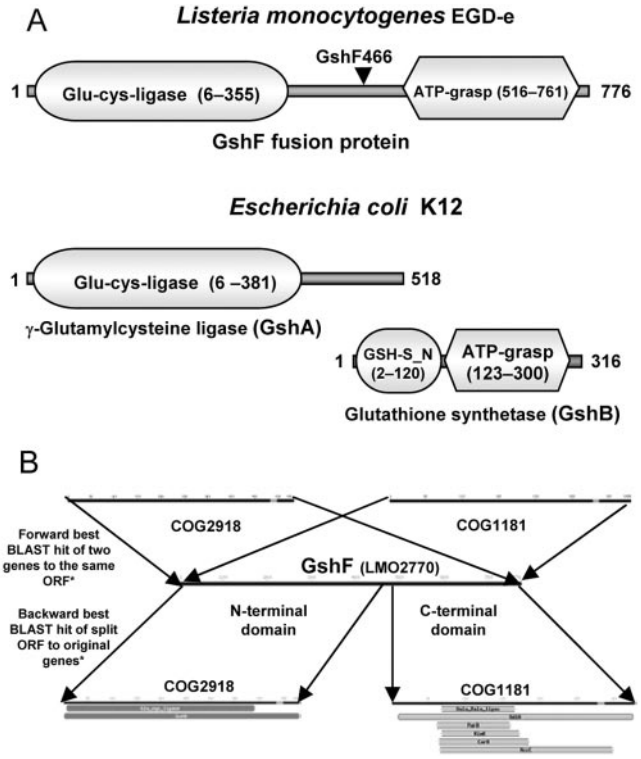

FIG. 1. (A) Schematic representation of the domain architecture of the *L. monocytogenes* GshF 776 amino acid fusion protein (Lmo2770) and the *E. coli* K-12 GshA (γ-glutamylcysteine ligase) and GshB (glutathione synthetase) proteins. Domains are indicated by boxes; numbers within boxes show the amino residues that form the corresponding domains. GSH-S_N is the prokaryotic glutathione synthetase N-terminal domain (accession number PFO2951). The arrowhead shows the position of the plasmid pSGhs1 insertion in *L. monocytogenes* GshF466. (B) Putative gene fusion event in the formation of GshF-like proteins, constructed by using FusionDB (http://igs-server.cnrs-mrs.fr/FusionDB/), a database of bacterial and archaeal gene fusion events.

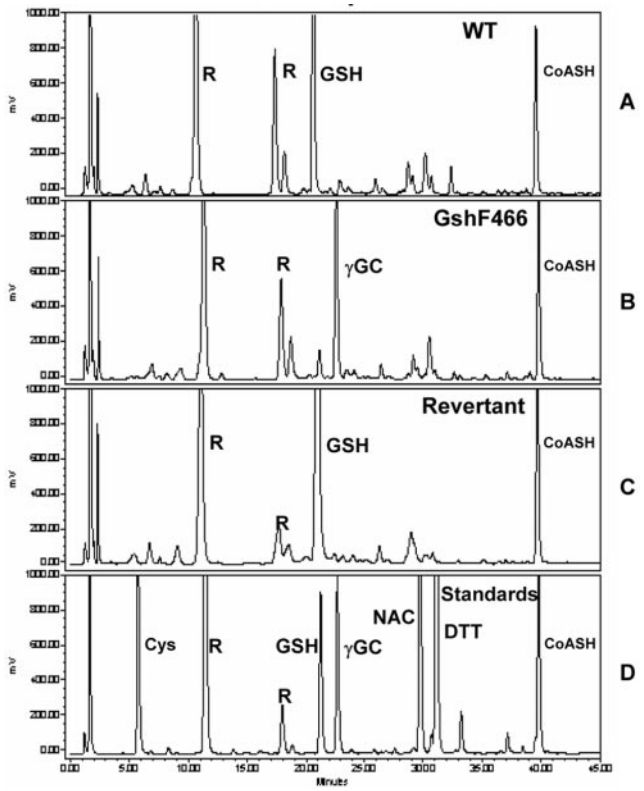

FIG. 2. HPLC analysis of the *L. monocytogenes* low-molecular-weight free thiols. Thiols were extracted and chromatographed from cultures of the wild type (WT) (A), the GshF466 mutant (B), and the GshF466 revertant (C) and chromatographed as described previously (20, 21), and they were compared with a chromatogram of a standard mixture of thiols (D) containing *N*-acetylcysteine (NAC), cysteine (Cys), coenzyme ASH (CoASH), and γ-glutamylcysteine (γGC). Peaks R were found in control samples in which thiols were blocked with *N*-ethylmaleimide prior to treatment with monobromobimane (data not shown) and were assumed to represent fluorescent components from the cells, reagent-derived components, or thiols having atypical reactivity. DTT, dithiothreitol.

cine, and Mg^{2+}ATP, and the reaction products were analyzed by HPLC (Table 2). GSH was made only when all three precursor amino acids were present in the reaction mixture. Omission of glycine resulted in formation of the intermediate γ-glutamylcysteine. GSH was also formed when the reaction mixture contained γ-glutamylcysteine and glycine. When either glutamate or ATP was omitted, no detectable cysteine-containing products were made. The Mg^{2+} required for γ-glutamylcysteine synthetase activity could be replaced by Mn^{2+}, a known property of this and related enzymes (1, 25). These results establish that the *L. monocytogenes* GshF protein functions as a multimodular enzyme system that has within a single polypeptide the catalytic sites required for substrate activation (adenylation) and peptide bond formation. In these respects, the GshF protein superficially resembles the multifunctional NRPS, such as L-α-aminoadipoyl-L-cysteine-D-valine (ACV) synthetase (2, 6, 20), that carry out stepwise assembly of small peptides on a single protein template. However, further characterization of the GshF-dependent reaction suggested that mechanistically it mimics the GshA-GshB two-enzyme system. One feature of NRPS is the absence of soluble peptide intermediates. In the system described here γ-glutamylcysteine was detected as a free intermediate in the synthesis of GSH. A second feature of NRPS is the involvement of a 4′-phosphopantetheine cofactor in the elongation process. We were unable to show the need for any cofactor other than ATP in the GshF-dependent reaction. A further difference between NRPS

TABLE 2. γ-Glutamylcysteine ligase and glutathione synthetase activities of the *L. monocytogenes* GshF recombinant fusion protein[a]

Activity	Relative activity (%)	Sp act (nmoles/min/mg)
γ-Glutamylcysteine ligase		
Glutamate + cysteine	100	274.15
Glutamate	<0.01	<0.05
Cysteine	<0.01	<0.05
Glutathione synthetase		
γ-Glutamylcysteine + glycine	100	258.70
γ-Glutamylcysteine	<0.27	<0.71
Glycine	<0.23	<0.59
γ-Glutamylcysteine ligase + glutathione synthetase (complete reaction)		
Glutamate + cysteine + glycine	100	210.70
Glutamate + glycine	<0.43	<0.90
Cysteine + glycine	<0.47	<0.99

[a] Reactions were carried out in the presence of ATP and Mg^{2+}.

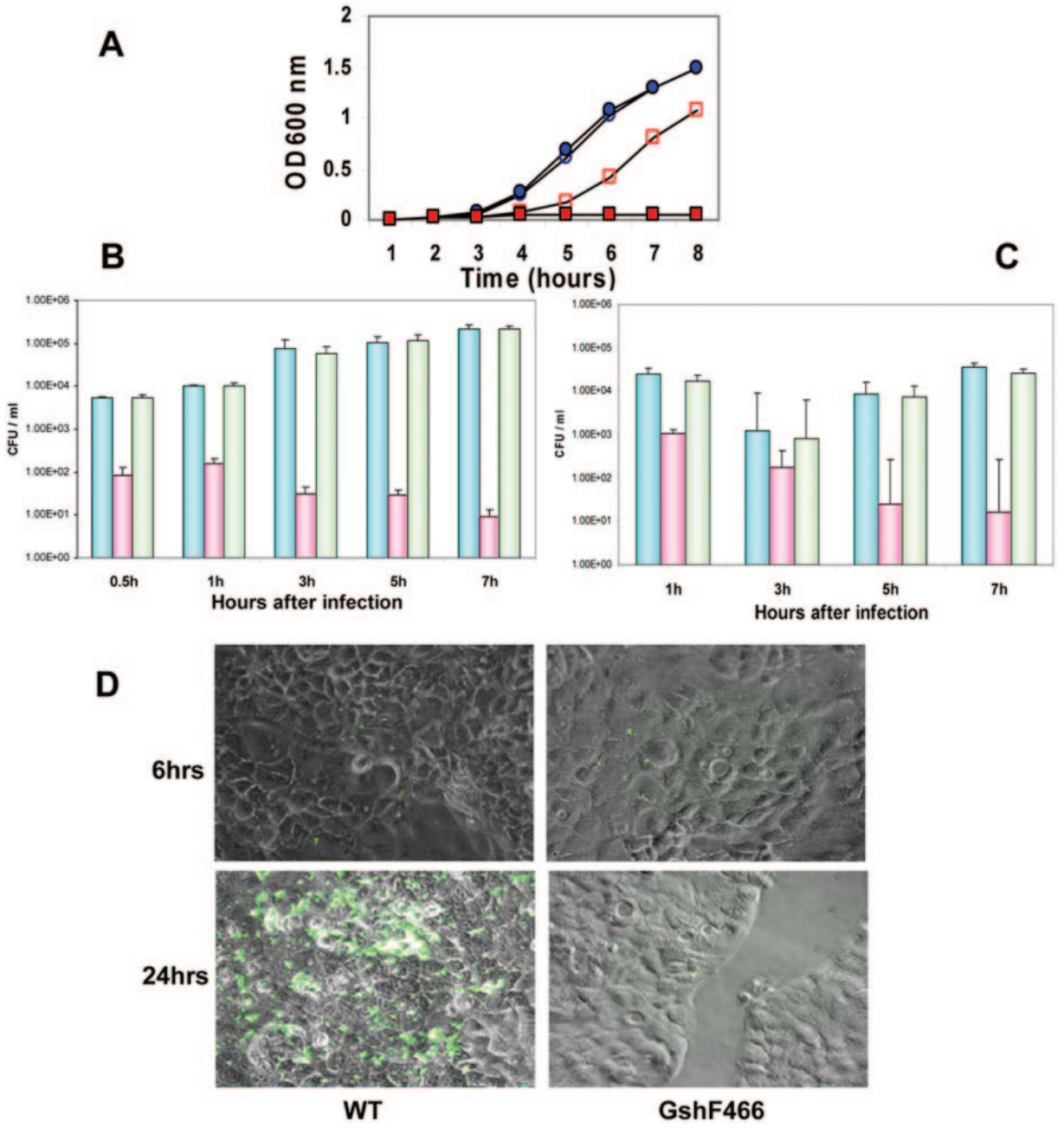

FIG. 3. Growth, survival, intracellular multiplication, and microscopic visualization of *L. monocytogenes* wild-type and mutant strains. (A) *L. monocytogenes* EGD-e (wild type) and the GshF466 insertion mutant were cultured at 37°C in tryptic soy broth containing 25 mM glucose with or without 2 mM reduced glutathione, and growth was monitored by determining the optical density at 600 nm (OD 600 nm). ●, EGD-e without GSH; ○, EGD-e with 2 mM glutathione; ■, Gsh466 without GSH; □, GshF466 with 2 mM glutathione. (B and C) Survival and intracellular multiplication of *L. monocytogenes* EGD-e (wild type), the Gsh466 insertion mutant, and the Gsh466 revertant in J774 mouse macrophage-like cells (B) and Caco-2 enterocyte-like cells (C). Multiplicities of infection of 1 and 10 bacteria per cell were used for J774 and Caco-2 cells, respectively. At 30 min and 1 h after infection, aliquots of cells were washed twice with phosphate-saline buffer to remove adherent bacteria, the infected cells were lysed, and the numbers of viable bacteria were determined and expressed in CFU/ml. Gentamicin (10 μg/ml) was added to the remaining cells to kill extracellular bacteria, and at 3, 5, and 7 h postinfection intracellular bacteria were recovered and enumerated. The graph shows average numbers of CFU/ml from four independent experiments. (D) Microscopic visualization of infection of *L. monocytogenes* wild-type strain EGD-e (WT) and the GshF466 mutant in Caco-2 cells. Bacteria were transformed with plasmid pLSV16-P*actA-gfp*, which expresses the *gfp* gene (encoding the green fluorescent protein) under the control of the *actA* promoter following entrance of the bacterium into the cytosol of an infected host cell (13). Caco-2 cells were infected as described above. At 6 h and 24 h postinfection, the infected cell monolayers were inspected by phase-contrast and fluorescence microscopy, and the micrographs were overlaid electronically.

TABLE 3. Sensitivity of the *L. monocytogenes* wild type, the GshF466 mutant, and the GshF466 revertant to hydrogen peroxide, diamide (azodicarboxylic acid bis[dimethylamide]), and *tert*-butyl alcohol hydroperoxide on BH1 agar plates

Strain	Diam of inhibition zone (mm)[a]											
	Hydrogen peroxide				Diamide				*tert*-Butyl alcohol hydroperoxide			
	5 μmol	7 μmol	9 μmol	13 μmol	2.5 μmol	3.5 μmol	5 μmol	7.5 μmol	40 μmol	60 μmol	77 μmol	115 μmol
Wild type	12–14	17–18	21–22	26–26	8–9	13–14	17–18	19–20	0	9–10	16–17	22–23
GshF466	17–18	22–23	24–25	29–30	12–13	16–17	20–21	24–25	2–3	>20[b]	>25[b]	>30[b]
Revertant	10–11	16–18	19–21	22–23	9–10	13–14	17–18	19–20	0	8–10	15–16	21–22

[a] The diameters of the zones of inhibition are the diameters obtained after incubation at 37°C for 24 h.
[b] The diameter of the inhibition zone could not be determined precisely because the zones merged.

and peptide synthetases such as γ-glutamylcysteine synthetase, D-alanine:D-alanine ligase, and glutathione synthetase is that the former enzymes activate acyl groups via aminoacyl-AMP intermediates, whereas the latter enzymes do so via phosphoryl transfer, yielding aminoacyl phosphate intermediates with cleavage of ATP to ADP. We used a spectrophotometric assay in which ADP formation is coupled with pyruvate kinase and lactate dehydrogenase (29) to show that ADP is indeed released in the complete reaction with purified enzyme.

Growth and survival of a GshF mutant defective in glutathione synthesis are severely impaired in mouse macrophages and Caco-2 enterocyte-like cells. *L. monocytogenes* is a serious food-borne human and animal pathogen that primarily affects pregnant women, newborns, and adults with weakened immune systems. It invades and multiplies in various host cells, among which are professional phagocytes, including macrophages (reviewed in reference 35). To assess the effect of abolishing GSH synthesis on growth, we first compared propagation of *L. monocytogenes* EGD-e (wild type) and the GshF466 mutant in TSB glutathione-deficient broth (Fig. 3A). The growth of the GshF466 mutant was markedly impaired, and the defect could be partially suppressed by addition of 2 mM reduced glutathione to the medium. To assess the ability of *L. monocytogenes* to survive and multiply within eukaryotic host cells, cultures of the wild type, the GshF466 mutant, and the GshF466 revertant were inoculated into a mouse macrophage-like cell line (J774), and viability was determined at various times after infection (Fig. 3B). At 30 min postinfection the number of viable intracellular bacteria recovered from macrophages was ~65-fold less with the GshF466 mutant (which was unable to synthesize GSH) than with the wild-type strain. In the next 30 min the number of intracellular mutant bacteria increased very slightly compared to the number of wild-type bacteria and then decreased steadily during the next 6 h. Thus, the GshF466 mutant was clearly unable to effectively cope with the oxidative burst mounted by the macrophages, which commenced within seconds after bacterial uptake.

We observed a similar behavior when Caco-2 enterocyte-like cells were infected with the wild-type and mutant strains (Fig. 3C); however, the initial reduction in the number of viable bacteria was demonstrably lower. The intracellular fate of the infecting bacteria was also monitored by fluorescence microscopy (Fig. 3D). To do this, *L. monocytogenes* wild-type and mutant bacteria were transformed with a plasmid (pLSV16-P*actA-gfp*) that expresses the green fluorescent protein under the control of the *actA* promoter, but only when the bacteria escape from the phagosome into the cytosol of an infected eukaryotic host cell (5). The low number of culturable fluorescent bacteria observed in the GshF466 mutant-infected cells compared with the wild-type-infected cells most likely reflects a defect in the propagation of the GshF466 mutant and is consistent with the slow growth of the GshF466 mutant in broth culture. However, we cannot exclude the possibility that the GshF466 mutant may also be impaired in its invasiveness, an issue currently under investigation. Furthermore, since the cytosol of mammalian cells contains significant amounts of glutathione (up to 5 mM), our results show that *L. monocytogenes* cannot efficiently take up GSH within the cell. Other experiments, in which cultures of the wild type and GshF466 mutant were exposed to hydrogen peroxide, diamide (a thiol-specific oxidant [15]), and τ-butyl hydroperoxide showed that in each case the growth of the mutant was impaired and that the effect was most pronounced with the organic peroxide (Table 3). These findings support a role for GSH in eliminating reactive oxygen species. In these experiments, as well as in the infection studies with macrophages, the GshF466 revertant behaved like the wild-type strain.

GshF-like fusion proteins are present mainly in gram-positive and gram-negative mammalian pathogens. Our results demonstrate the existence in *L. monocytogenes* of a multimodular fusion protein that specifies both γ-glutamylcysteine ligase and glutathione synthetase catalytic activities and enables GSH synthesis. We identified ORFs coding for proteins that are closely related to the listerial GshF protein in the genomes of all the gram-positive bacteria that we previously documented as organisms that produce GSH. In addition to *L. monocytogenes*, these organisms are *Streptococcus mutans*, *Streptococcus agalactiae*, *Enterococcus faecalis*, and *Enterococcus faecium*. The proteins encoded by these ORFs are presumably responsible for GSH synthesis in these bacteria. Preliminary studies indicated that GSH inhibits the γ-glutamylcysteine ligase activity of *L. monocytogenes* GshF by a feedback mechanism similar to that in *E. coli* (13). Interestingly, whereas some gram-positive bacteria are able to synthesize GSH (23), others can acquire it from the growth medium (33, 39). In this context, of the bacteria listed above that possess *gshF*-like genes, *S. mutans* has been reported to be able to transport glutathione into the cell, whereas *S. agalactiae* and *E. faecalis* import GSH at negligible or markedly lower rates (30). Further work should clarify if GSH synthesis and uptake in these bacteria are coregulated.

How prevalent are *gshF* fusion genes among bacteria? In addition to the four previously reported bacteria, *L. monocytogenes*, *L. innocua*, *C. perfringens*, and *P. multocida*, we iden-

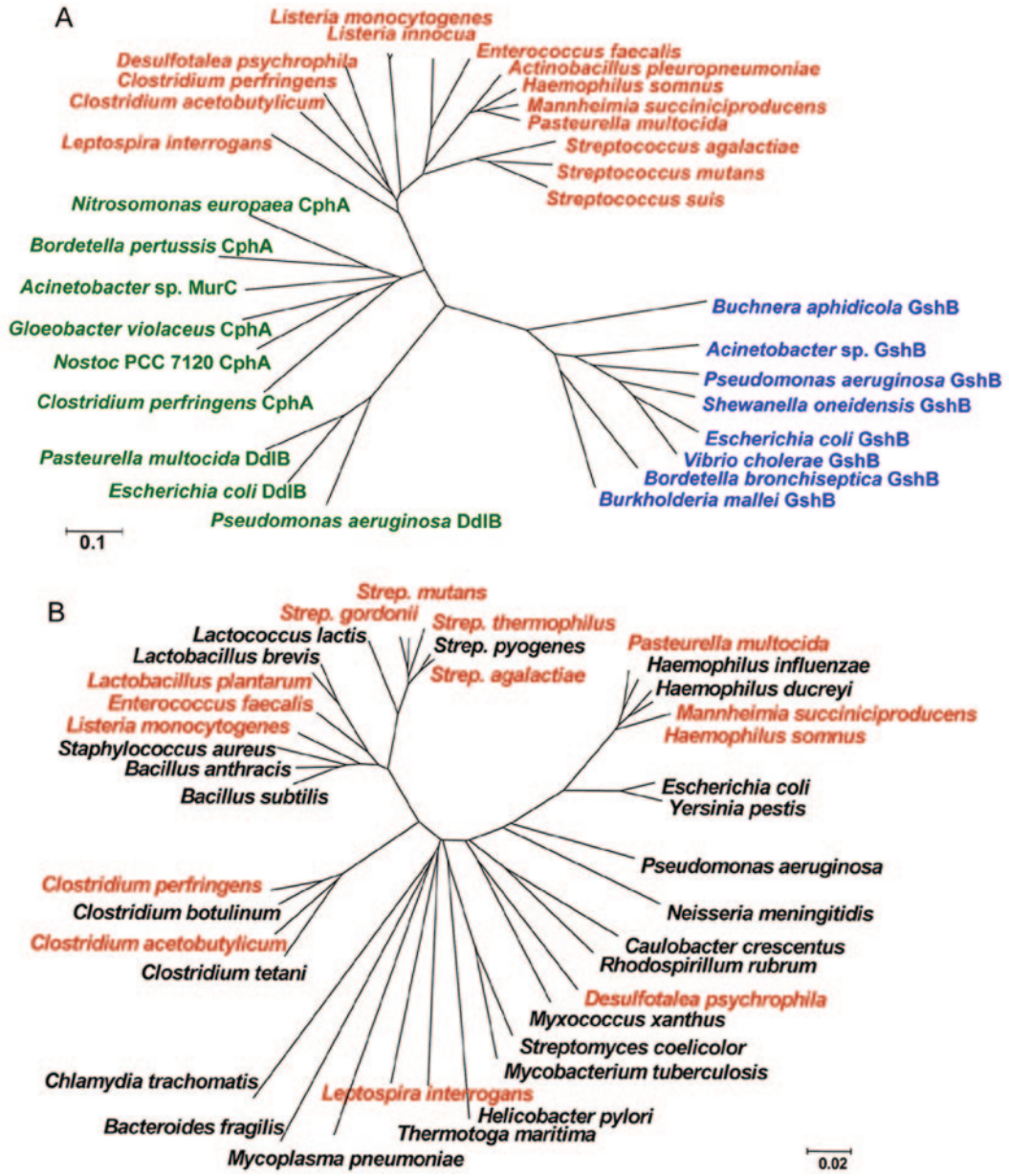

FIG. 4. Phylogeny of GshF fused proteins inferred by the minimum-evolution method. (A) GshF C-terminal domain sequences mapped on a tree of selected bacterial GshB and ATP-grasp protein sequences. Red, bacteria containing *gshF* genes encoding putative GshF fusion proteins; blue, bacteria containing genes encoding GshB proteins (glutathione synthetase); green, bacteria containing genes encoding ATP-grasp proteins (MurC, UDP-*N*-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase, DdlB, D-alanine:D-alanine ligase, CphA, cyanophycin synthetase). *L. interrogans* and *C. acetobutylicum* contain separate ORFs, corresponding to the N- and C-terminal domains, that overlap or are immediately adjacent to one another, respectively. (B) GshF fusion proteins mapped on a universal tree of bacterial 16S rRNA sequences indicated as follows: red, bacteria containing *gshF* genes encoding putative GshF fusion proteins; black, bacteria lacking *gshF* genes. In *L. interrogans* and *C. acetobutylicum* the *gshA* and *gshB* genes overlap and are adjacent, respectively. The protein sequences used were obtained from the GenBank and Swiss-Prot databases.

tified ORFs in the genomes of 16 more bacteria (out of some 400 different species that were examined) that are predicted to encode GshF-like proteins. These organisms are *S. agalactiae*, *Streptococcus gordonii*, *S. mutans*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, *Streptococcus suis*, *Streptococcus thermophilus*, *Streptococcus uberis*, *E. faecalis*, *E. faecium*, and *Lactobacillus plantarum* (gram-positive bacteria); *Actinobacillus pleuropneumoniae*, *Actinobacillus actinomycetemcomitans*, *Haemophilus somnus*, *Mannheimia succiniciproducens* (γ-proteobacteria); and *Desulfotalea psychrophila* (δ-proteobacterium). In *Leptospira interrogans* (a member of the spirochetes that diverged early in bacterial evolution), the *gshA* and ATP-grasp genes overlap, while in *Clostridium acetobutylicum* (a gram-positive bacterium) they are immediately adjacent to one other. In all the other bacteria examined the *gshA* and *gshB* genes were unlinked. We noted that the genomes of all of the bacteria containing a *gshF*-like gene are annotated as containing a gene coding for γ-glutamylcysteine ligase, but none are

annotated as containing a gene for glutathione synthetase. Not surprisingly, with the single exception of *C. acetobutylicum*, all of the bacteria containing a *gshF*-like gene also contain a gene coding for glutathione reductase, whose function is to maintain glutathione in its reduced state.

Gsh fusion proteins probably evolved by domain recruitment and were spread by lateral gene transfer. Finally, we point out that GshF-like trees obtained by protein sequence clustering suggest that a complex pattern of gene transfer events occurred during evolution. The GshF alignment for the C-terminal domain, consisting of 14 members, together with selected members of the bacterial glutathione synthetase GshB family of proteins (with which they exhibit no significant sequence similarity) and several members of the ATP-grasp family of proteins that were recognized in a BLAST search using the GshF C-terminal domain as proteins that exhibit moderate similarity, yielded the tree shown in Fig. 4A. Trees constructed by the protein distance (neighbor-joining and minimum-evolution) methods yielded essentially the same topologies. Surprisingly, bacteria harboring *gshF* genes are unusually placed in a highly supported group in which gram-positive species of streptococci, *Listeria*, and enterococci are clustered together with the gram-negative organisms *H. somnus*, *P. multocida*, *M. succiniciproducens*, *A. actinomycetemcomitans*, and *D. psychrophila.* Within each cluster, composed of GshFs and GshBs, sequence homology is clearly detected, whereas the homology between the clusters is questionable. When the N-terminal domains of GshF proteins and the entire GshA protein sequence were aligned and used to construct a tree, clustering of gram-negative and gram-positive GhsF proteins was also found, and a similar tree was obtained when the entire GshF protein sequence was used (data not shown). For comparison, when GshF-like proteins are assigned to a 16S rRNA tree (40), they are distributed in several distinct and well-separated gram-positive and gram-negative phylogenetic lineages (Fig. 4B). Several intriguing findings are evident from this analysis. First, in the species that were examined, *Listeria* and *Enterococcus* possess *gshF*-like genes and synthesize GSH, whereas species belonging to the related genera *Bacillus*, *Lactococcus*, and *Staphylococcus* lack *gshF*-like genes and do not produce GSH. Second, inspection of the genome databases of 12 streptococci revealed that 8 of them possess *gshF*-like genes (see above), while 4, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus equis*, and *Streptococcus mitis*, lack a *gshF*-like gene. Third, *H. somnus* (a gram-negative bacterium) contains a *gshF*-like gene, but the related organisms *Haemophilus ducrei* and *Haemophilus influenzae*, both of which import GSH from the medium, do not (36).

Although the origin of *gshF* genes is uncertain, a plausible scenario, first proposed by Copley and Dhillon (7), is one in which the part of the gene encoding the N-terminal domain, responsible for catalyzing peptide bond formation between glutamate and cysteine, originated from a bacterial *gshA* ancestor, while the part of the gene encoding the C-terminal domain, responsible for catalyzing ligation of glycine to γ-glutamylcysteine, was recruited from a member of the ATP-grasp superfamily, not necessarily from an authentic *gshB*. A similar hypothesis was recently proposed to account for the evolution of the α-L-glutamatate ligase homologs involved in the biosynthesis of methanogenic coenzyme F420 and tetrahydrosarcinapterin (18). The high degree of similarity in the C-terminal domain of GshF proteins in evolutionarily diverse prokaryotes (unpublished results) suggests that recruitment of the GshF ATP-grasp domain was a recent event. The remarkable distribution of *gshF* genes among bacteria leads us to speculate that the ancestral *gshF* gene subsequently spread among bacteria by horizontal gene transfer in a host-related environment, since nearly all the bacteria containing *gshF*-like genes are able to infect humans or domestic animals and with just one exception are mammalian pathogens. The odd exception is the psychrophilic bacterium *D. psychrophila*, a marine sulfate-reducing δ-proteobacterium that grows in permanently cold Arctic sediments (27).

Given that domain fusion can confer advantages on the host organism by providing increased efficiency of coupling of enzymatic reactions and by providing coordinate regulation of expression (19, 42), it is perhaps surprising that relatively few bacteria have evolved a multimodular enzyme to make GSH. A more extensive survey of the nature and distribution of GSH fusion proteins in bacteria employing a combination of bioinformatic, genetic, and physiological approaches would undoubtedly help elucidate some of the issues raised here.

In conclusion, here we provide positive evidence for the existence of a hitherto unknown and alternative route of glutathione biosynthesis. Bacteria containing this novel biosynthetic pathway include diverse gram-positive and gram-negative mammalian pathogens. A single multimodular fusion protein carries out complete synthesis of glutathione by integrating the two primary catalytic activities needed for synthesis. We propose that an important functional role of the fusion protein is to protect against oxidative stress. The evolutionary origin of the fusion protein appears to be the outcome of functional domain recruitment.

ACKNOWLEDGMENTS

We thank Matthias Wolf, Tobias Muller, and Tal Pupko for assistance with the phylogenetic analysis and David Gutnick for critical reading of the manuscript.

Parts of this work were funded by a grant (to S.G.) from the German Academic Exchange Service and by grant 031U213B from the German Ministry for Education and Research.

ADDENDUM IN PROOF

While this paper was under review, Janowiak and Griffith reported the biochemical characterization of a similar fusion protein, GshAB, in *Streptococcus agalactiae* (B. E. Janowiak and O. W. Griffin, J. Biol. Chem. **280:**11829–11839, 2005).

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