JOURNAL OF BACTERIOLOGY, Apr. 2004, p. 2173–2178 Vol. 186, No. 7
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.7.2173–2178.2004

Multiple Formaldehyde Oxidation/Detoxification Pathways in *Burkholderia fungorum* LB400

Christopher J. Marx,[1]† Jonathan A. Miller,[1] Ludmila Chistoserdova,[2] and Mary E. Lidstrom[1,2]*

Department of Microbiology[1] and Department of Chemical Engineering,[2] University of Washington, Seattle, Washington 98195-2180

Received 1 September 2003/Accepted 30 December 2003

Burkholderia **species are free-living bacteria with a versatile metabolic lifestyle. The genome of *B. fungorum* LB400 is predicted to encode three different pathways for formaldehyde oxidation: an NAD-linked, glutathione (GSH)-independent formaldehyde dehydrogenase; an NAD-linked, GSH-dependent formaldehyde oxidation system; and a tetrahydromethanopterin-methanofuran-dependent formaldehyde oxidation system. The other *Burkholderia* species for which genome sequences are available, *B. mallei*, *B. pseudomallei*, and *B. cepacia*, are predicted to contain only the first two of these pathways. The roles of the three putative formaldehyde oxidation pathways in *B. fungorum* LB400 have been assessed via knockout mutations in each of these pathways, as well as in all combinations of knockouts. The resulting mutants have the expected loss of enzyme activities and exhibit defects of varying degrees of severity during growth on choline, a formaldehyde-producing substrate. Our data suggest that all three pathways are involved in formaldehyde detoxification and are functionally redundant under the tested conditions.**

The lifestyle of free-living organisms involves many challenges related to both seasonal and sudden changes in nutrient supply, temperature, salinity, etc., and in some cases it appears that a correlation exists between the versatility of the lifestyle and the genome size (8, 30). Larger genome sizes are correlated not only with the variety of the functions encoded but also with redundancy (2, 29), which has been implicated as playing a significant role in the genetic robustness of organisms (11, 30). Two major types of functional redundancy are known: the presence of (multiple) gene paralogs with overlapping functions and the presence of nonhomologous biochemical pathways that fulfill similar functions. These two kinds of genetic redundancy have been extensively studied in eukaryotes, and currently, the second type of redundancy appears to play a major role in genetic robustness (11, 16, 35). Little is known about the role of functional redundancy in prokaryotes. The recent emergence of the complete genomic sequences for a number of free-living microbes permits approaches for addressing this question.

One of the important biochemical necessities in life is the ability to detoxify highly toxic aldehydes, intermediates of many biochemical pathways, of which formaldehyde is the most toxic (4, 9). Four different pathways for formaldehyde detoxification are known in bacteria, encoded by unrelated or distantly related genes. The best characterized is the pathway involving glutathione (GSH)-dependent NAD-linked formaldehyde dehydrogenase (GSH-FDH) and formyl-GSH hydrolase (FGH), which is found in both prokaryotes and eukaryotes (10, 14, 15, 17, 25, 27). An FDH that does not require GSH for its activity has been characterized from *Pseudomonas putida* (31), and homologs of this enzyme are also widespread (30). However, the physiological function of this enzyme has not been tested by mutation. A third pathway for formaldehyde detoxification involves the enzymes that are characteristic of the ribulose-monophosphate cycle methylotrophs: hexulose phosphate synthase (HPS) and hexulose phosphate isomerase (HPI) (1, 7). More recently it has been shown that these are also present in heterotrophs, and it has been suggested that they play a role in formaldehyde handling (37). The fourth pathway is carried out by "archaeal" enzymes and is linked to the archaeal cofactors tetrahydromethanopterin (H_4MPT) and methanofuran (MFR) (6). This pathway has so far been identified only in methylotrophic bacteria and has been characterized in detail in one methylotroph, *Methylobacterium extorquens* AM1 (12, 20, 21, 23, 24, 32–34). Based on mutant analysis in this organism, 18 genes have been implicated in this pathway, 16 that have homologs in archaea and 2 that do not (5, 6, 12, 21). Some methylotrophs have multiple formaldehyde oxidation pathways, and at least in some cases these do not appear to be redundant (7).

In this work it is shown that *Burkholderia fungorum* LB400 contains three of the four known routes for formaldehyde oxidation, including the H_4MPT-MFR-dependent pathway. These pathways have been shown to be nonhomologous and redundant under the conditions tested, and each is differentially involved in growth on choline, a formaldehyde-generating substrate.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. *B. fungorum* LB400 (3) was grown at 30°C in a minimal medium (13) supplemented with one of the following carbon sources: succinate (0.4% [wt/vol]), choline (0.2% [wt/vol]), and citrate (0.2% [wt/vol]). To test for growth on methylotrophic substrates, methanol (0.1 to 0.5% [vol/vol]) or

* Corresponding author. Mailing address: Department of Chemical Engineering, University of Washington, Box 352180, Seattle, WA 98195-2180. Phone: (206) 616-5282. Fax: (206) 616-5721. E-mail: lidstrom@u.washington.edu.

† Present address: Michigan State University, East Lansing, MI 48824-4320.

TABLE 1. *B. fungorum* LB400 strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
B. fungorum LB400	Wild type	3
CM359.1	Δ*mtdB*	This study
CM359K.1	Δ*mtdB*::*kan*	This study
CM359-363.1	Δ*mtdB* Δ*flhA*	This study
CM359-363K.1	Δ*mtdB* Δ*flhA*::*kan*	This study
CM359-363-409K.1	Δ*mtdB* Δ*flhA* Δ*fdhA*::*kan*	This study
CM359-409K.1	Δ*mtdB* Δ*fdhA*::*kan*	This study
CM363.1	Δ*flhA*	19
CM363K.1	Δ*flhA*::*kan*	19
CM363-409K.1	Δ*flhA* Δ*fdhA*::*kan*	This study
CM409K.1	Δ*fdhA*::*kan*	This study
Plasmids		
pCM157	Cre expression plasmid	19
pCM184	Allelic-exchange vector	19
pCM356	pCR2.1 with *mtdB* upstream	This study
pCM357	pCR2.1 with *mtdB* downstream	This study
pCM358	pCM184 with *mtdB* downstream	This study
pCM359	pCM358 with *mtdB* upstream	This study
pCM363	Construct for Δ*flhA*::*kan* mutations	19
pCM406	pCR2.1 with *fdhA* upstream	This study
pCM407	pCR2.1 with *fdhA* downstream	This study
pCM408	pCM184 with *fdhA* upstream	This study
pCM409	pCM408 with *fdhA* downstream	This study
pCR2.1	PCR cloning vector	Invitrogen

methylamine (0.5% [wt/vol]) was added to the same minimal medium in agar plates. Growth on methane or methanethiol was tested under a substrate-air atmosphere (50:50 and 20:80, respectively). In addition, a number of other potential methylotrophic substrates were tested in agar plates at 10 to 20 mM concentrations, including dimethyl sulfide, dimethyl sulfoxide, betaine, sarcosine, and dimethylglycine. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani medium (18). Antibiotics were supplied at the indicated concentrations (micrograms per milliliter): ampicillin (50), chloramphenicol (10), kanamycin (*E. coli*, 50; *B. fungorum* LB400, 20 on solid medium and 10 in liquid), and tetracycline (10).

Sequence analysis. The draft genomic sequences of *B. fungorum* LB400 and *Burkholderia mallei* have been assessed at www.jgi.doe.gov/JGI_microbial/html/index.html. The genomic sequences of *Burkholderia cepacia* and *Burkholderia pseudomallei* have been assessed at http://www.sanger.ac.uk/Projects/B_cepacia. The sequence of *Methylococcus capsulatus* Bath has been assessed at www.tigr.org/tdb/mdb/mdbinprogress.html. The sequences of the archaeal genes for formaldehyde oxidation in *M. extorquens* AM1 are available at http://www.ncbi.nlm.nih.gov under accession numbers AF032114, AY117134, and AY093431. To search the genomes of the four available *Burkholderia* species for the presence of formaldehyde handling functions, BLAST engines available at the genome sites listed were used. The following amino acid sequences were used as queries: the GSH-linked FDH and FGH from *Paracoccus denitrificans* (GenBank accession numbers L36327 and U34346, respectively), the GSH-independent FDH from *P. putida* (GenBank accession number D21201), the HPS and HPI from *Methylomonas aminofaciens* (GenBank accession number AB026428), and all 18 polypeptides involved in H_4MPT-MFR-linked formaldehyde oxidation from *M. extorquens* AM1 (GenBank accession numbers are listed in reference 5).

Mutant generation. *B. fungorum* LB400 mutants bearing deletions in *mtdB* (H_4MPT-dependent formaldehyde oxidation) and *fdhA* (GSH-independent formaldehyde oxidation) were generated using the recently designed broad-host-range *cre-lox* system for antibiotic marker recycling (19). The constructs for allelic exchange were created by PCR amplification of regions of approximately 0.5 kb immediately upstream and downstream of *mtdB* and *fdhA*. These PCR products were first sequenced to ensure that no mutations were introduced and then cloned into pCR2.1 (Invitrogen) to yield pCM356 and pCM357 (*mtdB*) and pCM406 and pCM407 (*fdhA*). The construct for obtaining Δ*mtdB*::*kan* mutants was generated by introducing the 0.6-kb SacII-SacI fragment from pCM357 into the same sites of pCM184 to produce pCM358, into the same site of which the 0.5-kb EcoRI fragment from pCM356 was introduced, resulting in pCM359. The construct for obtaining Δ*fdhA*::*kan* mutants was generated by introducing the 0.5-kb BamHI-NdeI fragment from pCM406 between the BglII and NdeI sites of pCM184 to produce pCM408, into the same sites of which the 0.6-kb SacII-SacI fragment from pCM407 was cloned, yielding pCM409. These donor plasmids were conjugated into *B. fungorum* LB400 by use of the *E. coli* helper strain S17-1 (28), and null mutants were identified as previously described (19). Double-crossover recombination events were confirmed by diagnostic PCR. To generate mutants deficient in more than one formaldehyde oxidation pathway, additional mutations were introduced as described above in unmarked deletion backgrounds generated by Cre-mediated recombination as described previously (19).

Enzyme assays. The activities of GSH-dependent and GSH-independent FDHs were measured in the following reaction mixture: 50 mM Tris-HCl (pH 8.0), 1 mM NAD, 10 mM formaldehyde, and 2 mM GSH for the former. To discriminate between the two enzymes, they were visualized in isoelectrofocusing gels (PhastSystem; Amersham) by activity staining (data not shown). The activity staining mixture was the same as that described above with phenazine methosulfate (0.5 mM) and nitroblue tetrazolium (0.1 mM) added for color development. The activities of methylene-H_4MPT dehydrogenase and methenyl-H_4MPT cyclohydrolase were determined as described in references 32 and 23, respectively. Protein concentrations were determined by spectrophotometric assay (36).

RT-PCR analysis. RNA was isolated from 3-ml cultures of *B. fungorum* LB400 grown on choline or citrate to an optical density at 600 nm of 0.4, with the Qiagen RNeasy kit, including the DNase I (Qiagen) treatment. The preparations were repeatedly treated with DNase I (ZymoResearch, Orange, Calif.) and further purified and concentrated using the ZymoResearch RNA purification kit. First-strand cDNA synthesis was performed using the reverse transcription-PCR (RT-PCR) kit (avian myeloblastosis virus) from Roche, from a primer (Fae1) complementary to the 3′ part of the *fae* genes, followed by 30 cycles of PCR at the annealing temperature of 55°C from Fae1 and Fae2 (complementary to the 5′ part of *fae*).

Phenotypic analyses of *B. fungorum* LB400 strains. Growth of wild-type *B. fungorum* LB400 and the mutant strains was monitored on both solid and liquid media, with all phenotypic analyses performed in at least two replicates. Growth on solid medium was determined by comparing the sizes and rates of colony formation. Growth curves in minimal media were obtained using 30 ml of culture in 250-ml flasks shaken at 250 rpm. Inoculum was obtained by pelleting exponentially growing cultures by centrifugation and resuspending cells in fresh growth medium to an optical density at 600 nm of 0.1.

RESULTS

Multiple formaldehyde oxidation pathways are encoded in the genomes of *Burkholderia* species. To assess the presence of genes encoding putative formaldehyde oxidation pathways in the four *Burkholderia* genomes, similarity searches with polypeptide query sequences indicative of each of the four known pathways were carried out as described in Materials and Methods. Putative genes for the GSH-linked pathway were identified in all four *Burkholderia* genomes by the high similarity of their encoded polypeptides with the queries. The two genes were found clustered together in all four genomes, in the order *flhA-fghA*. The gene for the GSH-independent FDH (*fdhA*) was similarly identified in all four genomes. None of the genomes contained the genes for HPS and HPI, and only one genome, of *B. fungorum* LB400, contained the genes for the archaeal pathway for formaldehyde oxidation (Fig. 1). Homologs of 17 of the 18 genes implicated in the archaeal formaldehyde oxidation pathway in *M. extorquens* AM1 (5) were detected in *B. fungorum* LB400, and as in *M. extorquens* AM1, they were found arranged in one cluster, with the gene order partially conserved between the two organisms (Fig. 2). One gene involved in the H_4MPT-linked formaldehyde oxidation in *M. extorquens* AM1, *dmrA*, which is suggested to encode the final reaction in the biosynthesis of H_4MPT (21), has no homolog in the *B. fungorum* LB400 genome. It is possible that this function could be encoded by a nonhomologous gene. The presence of the archaeon-like pathway in a bacterium not

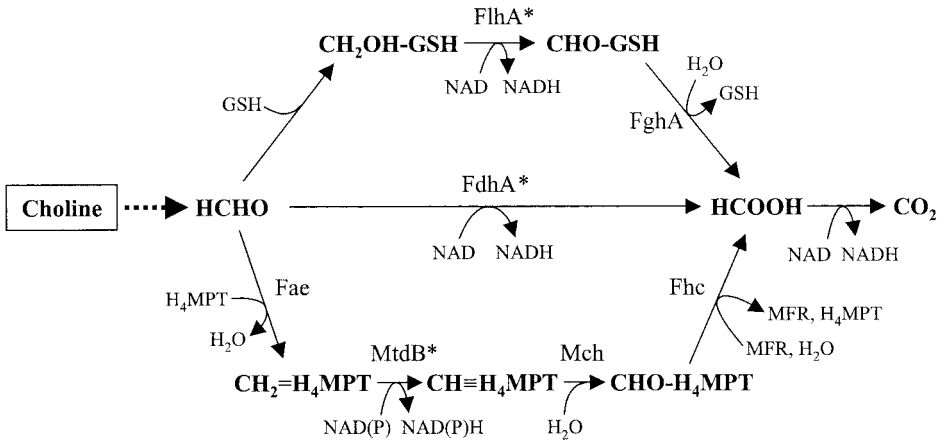

FIG. 1. Reactions of the three alternative pathways for formaldehyde oxidation in *B. fungorum* LB400. FlhA, GSH, NAD-linked FDH; FghA, FGH; FdhA, NAD-linked (GSH-independent) FDH; Fae, formaldehyde-activating enzyme; MtdB, NAD(P)-linked methylene-H_4MPT dehydrogenase; Mch, methenyl-H_4MPT cyclohydrolase; Fhc, formyltransferase-hydrolase complex. Genes subjected to mutation in this study are denoted by asterisks.

known to be a methylotroph prompted further study of its role in this bacterium.

The three pathways for formaldehyde oxidation are functional in *B. fungorum* LB400. The functionality of the three pathways potentially involved in formaldehyde oxidation was tested in *B. fungorum* LB400 by measuring specific enzyme activities characteristic of each pathway. As is known from previous work, formaldehyde detoxification pathways are often not expressed constitutively in bacteria but are induced in the presence of formaldehyde or formaldehyde-producing substrates (25, 37). Therefore, we searched the genome of *B. fungorum* LB400 for genes that potentially encode formaldehyde-producing enzymes and/or pathways. No genes predicted to encode homologs of typical methylotrophic formaldehyde-generating systems, such as methanol dehydrogenase, methylamine dehydrogenase, halomethane degradation enzymes, or methane oxidation systems, are present in the genome of *B. fungorum* LB400, and accordingly, growth tests on methane, methanol, or methylamine were negative. A number of other methylated substrates were also tested, with negative results. The only obvious formaldehyde-producing metabolic pathway deduced from the *B. fungorum* LB400 genome was the pathway for oxidation of choline, via betaine aldehyde, betaine, dimethylglycine, and sarcosine, which produces two formaldehydes per betaine or choline (http://www.expasy.ch/cgi-bin/searchbiochem-index). Therefore, we tested *B. fungorum* LB400 for growth on choline and betaine and observed strong growth on both substrates. Thus, we used a formaldehyde-producing substrate, choline, and a non-formaldehyde-producing substrate, citrate, to test for the enzyme activities in question. The GSH-independent FDH was present at high levels in choline-grown cultures, while no activity was detected in citrate-grown cultures (Table 2). In contrast, the GSH-dependent FDH was present at moderate levels in citrate-grown cultures (Table 2). In wild-type cells grown on choline, the GSH-linked activity was masked by the higher activity of GSH-independent FDH, but tests in the FDH-negative mutant (see below) indicated that GSH-FDH is present in choline-grown cells at slightly elevated levels. To test for the functionality of the archaeal pathway, we measured activities of two key enzymes in the pathway, methylene-H_4MPT dehydrogenase (MtdB) and methenyl-H_4MPT cyclohydrolase (Mch). Neither was de-

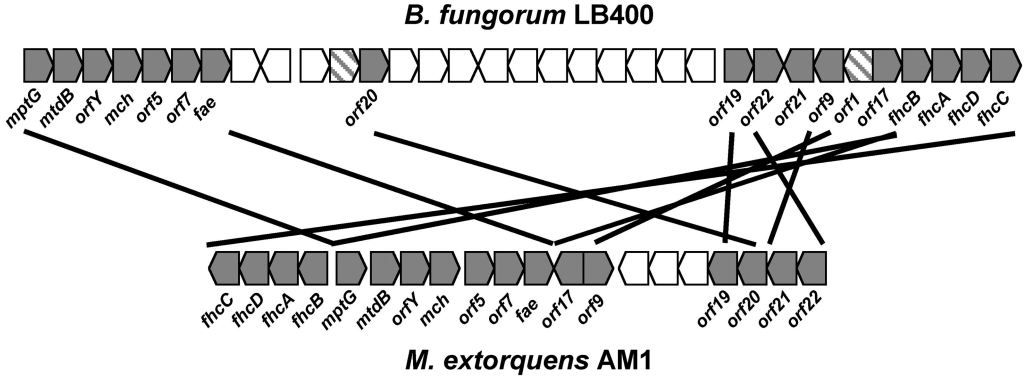

FIG. 2. Alignment of the archaeal gene clusters from *B. fungorum* LB400 and *M. extorquens* AM1. Gray symbols connected by lines, gene homologs of archaeal nature; striped symbols, genes of *B. fungorum* LB400 having homologs in *Archaea* but not in *M. extorquens AM1*; open symbols, genes not considered in this study.

TABLE 2. Enzyme activities measured in wild-type and mutant strains of *B. fungorum* LB400 grown on citrate and choline.

Strain	Sp act (mU)[a]							
	Citrate				Choline[b]			
	FDH[c]	FDH(G)[d]	MtdB	Mch	FDH[c]	FDH(G)[d]	MtdB	Mch
Wild type	<1	100	<1	<1	800	750	20	20
flhA	<1	<1	ND[e]	<1	700	750	ND	22
fdhA	<1	30	ND	<1	<1	200	ND	23
mtdB	<1	45	<1	<1	650	700	<1	25
flhA fdhA	<1	<1	<1	<1	25	25	ND	22
flhA mtdB	<1	<1	ND	<1	660	590	ND	24
mtdB fdhA	<1	45	ND	<1	<1	145	ND	23
flhA fdhA mtdB	<1	<1	ND	<1	<1	<1	ND	25

[a] Specific activities are in milliunits (nanomoles of substrate converted per minute per milligram of protein). Assays were carried out in triplicate, and activity values agreed between the replicates within 15%.

[b] The triple mutant is severely defective for growth on choline. To test for enzyme activities normally induced during growth on choline, cells were grown in citrate-supplemented medium, pelleted, and incubated in the presence of choline overnight.

[c] FDH assay with no GSH added (see Materials and Methods).

[d] FDH assay with GSH added.

[e] ND, not determined.

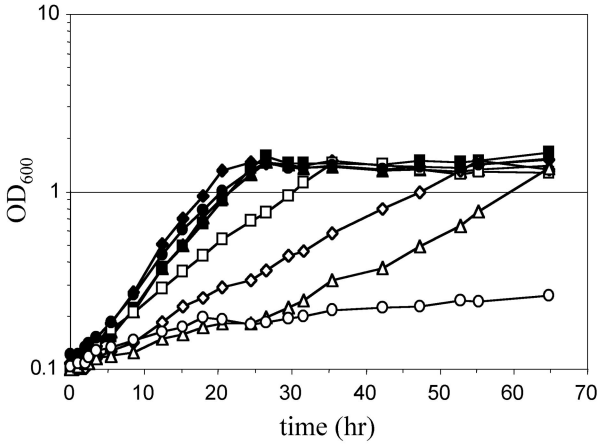

FIG. 3. Growth of *B. fungorum* LB400 strains on choline. The strains with GSH-independent FDH activity are shown by filled symbols: squares (wild type), diamonds (*mtdB*::Km), triangles (*flhA*::Km), and circles (*mtdB flhA*::Km). Strains lacking GSH-independent FDH activity are shown by open symbols: squares (*fdhA*), diamonds (*mtdB fdhA*::Km), triangles (*flhA fdhA*::Km), and circles (*mtdB flhA fdhA*::Km). OD_{600}, optical density at 600 nm.

tectable in citrate-grown cells, while both were present at low but significant levels in choline-grown cells (Table 2). In addition, we tested for the expression of a gene (*fae*) encoding another key enzyme of the archaeal pathway, formaldehyde-activating enzyme, which directly binds formaldehyde, via RT-PCR. *fae*-specific mRNA was detected in both choline-grown and citrate-grown cells but was more abundant in choline-grown cells (data not shown).

Mutant analysis suggests that all three pathways contribute to formaldehyde tolerance. To test the potential physiological functions of the three formaldehyde oxidation pathways, mutants were generated that were defective in each of the pathways, defective in combinations of two different pathways, and defective in all three pathways. Deletions in the following genes were generated as described in Materials and Methods: *fdhA* to block the GSH-independent pathway, *flhA* to block the GSH-dependent pathway, and *mtdB* to block the archaeal pathway. All lesions were confirmed by diagnostic PCR (data not shown). As expected, the *fdhA* mutant lacked detectable GSH-independent FDH activity, and the *mtdB* mutant lacked detectable methylene-H_4MPT dehydrogenase activity (Table 2). The *flhA* single mutant lacked the GSH-dependent activity in citrate-grown cells, but no change in activities in choline-grown cells could be detected due to masking by FdhA. However, in the *flhA fdhA* double mutant, the GSH-dependent activity was reduced to low levels compared to those in the *fdhA* single mutant, demonstrating loss of FlhA activity in that case (Table 2). A low-level GSH-independent activity was also detected in choline-grown cells of the double *flhA fdhA* mutant, suggesting the presence of a fourth, unknown aldehyde oxidation system.

Growth characteristics of the mutants were tested on both citrate and choline and compared to those of the wild-type strain. All mutant strains grew on citrate at the same rate as did the wild-type strain (data not shown). Additionally, mutants defective in the GSH-linked pathway only or defective in the archaeal pathway only and the double mutant negative for both of these pathways displayed growth characteristics on choline similar to those of the wild-type strain (Fig. 3), suggesting that the GSH-independent FDH alone is sufficient to metabolize the formaldehyde produced during oxidation of choline. Growth of the mutant defective in the GSH-independent FDH was diminished compared to that of the wild-type strain, however, suggesting that this enzyme must play a significant role in formaldehyde oxidation. An even greater choline growth defect was observed for the double mutants lacking the GSH-independent FDH and either the archaeal pathway or the GSH-dependent FDH. These results indicate that these other two pathways can contribute to formaldehyde oxidation, but the contribution can be observed only in the absence of FDH. Finally, the mutant defective in all three formaldehyde oxidation systems exhibited the most dramatic growth defect on choline, resulting in almost complete arrest of growth.

DISCUSSION

Functional annotation of bacterial genomes and metabolic reconstruction based on such annotations is difficult when redundant pathways and/or enzymes are present that are predicted to perform a similar function. In some cases, multiple pathways are differentially regulated and are not physiologically redundant (22), while in other cases true functional redundancy exists, at least under laboratory growth conditions (11, 26). One physiologically critical function that often involves redundancy is formaldehyde detoxification, and in this work, formaldehyde oxidation pathways were assessed in metabolically versatile free-living *Burkholderia* strains. Genomic analysis of four species of *Burkholderia* revealed a potential for multiple formaldehyde oxidation pathways in all the species. Further studies of one of these strains, *B. fungorum* LB400, demonstrated the functional presence of three of the four known formaldehyde oxidation systems: the GSH-linked pathway carried out via two specific enzymes, GSH- and NAD-

linked FDH and FGH, encoded by the two genes tightly linked on the chromosomes of *Burkholderia* (*flhA-fghA*), the GSH-independent FDH pathway encoded by a single gene (*fdhA*), and the archaeal H_4MPT-MFR-linked pathway involving several linked genes (Fig. 2).

The function of the GSH-linked pathway for formaldehyde detoxification has been addressed previously for both prokaryotes and eukaryotes (14, 17, 25), and in the case of *P. denitrificans*, a facultative methylotrophic autotroph, the pathway was demonstrated to play a role in growth not only on C_1 compounds but also on choline (25). Therefore, a functional role of this pathway in choline utilization in *B. fungorum* LB400 was not surprising. However, the function of the GSH-independent FDH has not been tested by mutation in any strain, and we have confirmed a key role for this enzyme in formaldehyde detoxification. The predicted presence of this enzyme in all four *Burkholderia* species tested suggests that it may play a central role in formaldehyde metabolism in these bacteria.

In addition to the two pathways involving dehydrogenases of formaldehyde, an alternative, elaborate pathway is present in one of the *Burkholderia* species tested, *B. fungorum* LB400. It involves multiple C_1 transfer reactions similar to those of methanogenesis, and it utilizes the archaeal cofactors H_4MPT and MFR. Previously, the presence of this pathway has been reported only for methylotrophic proteobacteria. In methylotrophs, two distinct functions have been recognized for the pathway: a central role in energy generation during methylotrophic growth (6, 20, 21, 23, 32, 34) and an accessory role in formaldehyde detoxification (7, 20). However, in *B. fungorum* LB400 this pathway appears to play a minor detoxification role, at least during growth on choline. Since the attempts to discover a methylotrophic substrate for this strain were unsuccessful, it appears at this time to be a nonmethylotroph. The presence of the archaeal pathway in *B. fungorum* LB400 further expands the distribution of this pathway in the *Proteobacteria*. In methylotrophs, the enzyme activities of the H_4MPT-MFR-linked pathway are at much higher levels than are those in *B. fungorum* LB400. Low expression of the archaeal pathway in *B. fungorum* LB400 is consistent with the proposed minor role in formaldehyde detoxification. However, it is possible that this pathway might play a more significant role in growth on a yet unknown formaldehyde-generating substrate.

One known gene required for the H_4MPT-MFR-linked pathway in *M. extorquens* AM1 (*dmrA*) was not recognized in the genome. However, this gene is also missing from the genome of a gamma-proteobacterial methanotroph, *Methylococcus capsulatus*, suggesting that a functional alternative to *dmrA* must be present in this bacterium. One candidate for such a gene that shows homology to archaeal genes of unknown function is designated *orf1* in Fig. 2. This gene is found in the archaeal-like gene cluster in both *M. capsulatus* and *B. fungorum* LB400 but not in *M. extorquens* AM1.

The results presented here suggest that the GSH-independent FDH alone is capable of fulfilling the necessary formaldehyde detoxification function for growth on choline independently of the presence of the two other systems. This correlates with the high activity of the FdhA during growth on choline, compared to the activities of FlhA and MtdB, enzymes indicative of the GSH-dependent and the archaeal pathways, respectively. In the absence of the GSH-independent pathway, however, the two other pathways take over the role, with the GSH-linked pathway being more effective than the H_4MPT-MFR pathway in terms of supporting growth on choline. In the absence of all three pathways, *B. fungorum* LB400 is dramatically affected in its ability to use choline as a growth substrate. It is worth noting that even more redundancy must exist towards formaldehyde handling, as low FDH activity was detected in the triple mutant. This must be due to expression in this mutant of one or more other systems capable of formaldehyde oxidation at low levels.

ACKNOWLEDGMENTS

We are grateful to R. K. Thauer for a generous gift of H_4MPT and methenyl-H_4MPT and to Rebecca Parales and David Gibson for providing *B. fungorum* LB400.

This work was supported by grants from the NIH (GM58933) to M.E.L. and from the NSF (MCB-0131957) to M.E.L. and L.C.

REFERENCES

1. **Anthony, C.** 1982. The biochemistry of methylotrophs. Academic Press, London, United Kingdom.
2. **Bansal, A. K.** 1999. An automated comparative analysis of 17 complete microbial genomes. Bioinformatics **15:**900–908.
3. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. **1:**23–29.
4. **Chang, C. C., and M. E. Gershwin.** 1992. Perspectives on formaldehyde toxicity: separating fact from fantasy. Regul. Toxicol. Pharmacol. **16:**150–160.
5. **Chistoserdova, L., S.-W. Chen, A. Lapidus, and M. E. Lidstrom.** 2003. Methylotrophy in *Methylobacterium extorquens* AM1 from a genomic point of view. J. Bacteriol. **185:**2980–2987.
6. **Chistoserdova, L., J. A. Vorholt, R. K. Thauer, and M E. Lidstrom.** 1998. C_1 transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic archaea. Science **281:**99–102.
7. **Chistoserdova, L., L. Gomelsky, J. A. Vorholt, M. Gomelsky, Y. D. Tsygankov, and M. E. Lidstrom.** 2000. Analysis of two formaldehyde oxidation pathways in *Methylobacillus flagellatum* KT, a ribulose monophosphate cycle methylotroph. Microbiology **146:**3762–3769.
8. **Deppenmeier, U., A. Johann, T. Hartsch, R. Merkl, R. A. Schmitz, R. Martinez-Arias, A. Henne, A. Wiezer, S. Baumer, C. Jacobi, H. Bruggemann, T. Lienard, A. Christmann, M. Bomeke, S. Steckel, A. Bhattacharyya, A. Lykidis, R. Overbeek, H. P. Klenk, R. P. Gunsalus, H. J. Fritz, and G. Gottschalk.** 2002. The genome of *Methanosarcina mazei*: evidence for lateral gene transfer between bacteria and archaea. J. Mol. Microbiol. Biotechnol. **4:**453–461.
9. **Feron, V. J., H. P. Til, F. de Vrijer, R. A. Woutersen, F. R. Cassee, and P. J. van Bladeren.** 1991. Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment. Mutat. Res. **259:**363–385.
10. **Giese, M., U. Bauer-Doranth, C. Langebartels, and H. Sandermann, Jr.** 1994. Detoxification of formaldehyde by the spider plant (*Chlorophytum comosum* L.) and by soybean (*Glycine max* L.) cell-suspension cultures. Plant. Physiol. **4:**1301–1309.
11. **Gu, Z., L. M. Steinmetz, X. Gu, C. Scharfe, R. W. Davis, and W. H. Li.** 2003. Role of duplicate genes in genetic robustness against null mutations. Nature **421:**63–66.
12. **Hagemeier, C. H., L. Chistoserdova, M. E. Lidstrom, R. K. Thauer, and J. A. Vorholt.** 2000. Characterization of a second methylene tetrahydromethanopterin dehydrogenase from *Methylobacterium extorquens* AM1. Eur. J. Biochem. **267:**3762–3769.
13. **Harder, W., M. Attwood, and J. R. Quayle.** 1973. Methanol assimilation by *Hyphomicrobium* spp. J. Gen. Microbiol. **78:**155–163.
14. **Harms, N., J. Ras, W. N. Reijnders, R. J. van Spanning, and A. H. Stouthamer.** 1996. *S*-Formylglutathione hydrolase of *Paracoccus denitrificans* is homologous to human esterase D: a universal pathway for formaldehyde detoxification? J. Bacteriol. **178:**6296–6299.
15. **Hoog, J. O., J. J. Hedberg, P. Stromberg, and S. Svensson.** 2001. Mammalian alcohol dehydrogenase—functional and structural implications. J. Biomed. Sci. **8:**71–76.
16. **Kitami, T., and J. H. Nadeau.** 2002. Biochemical networking contributes more to genetic buffering in human and mouse metabolic pathways than does gene duplication. Nat. Genet. **32:**191–194.
17. **Lee, B., H. Yurimoto, Y. Sakai, and N. Kato.** 2002. Physiological role of the glutathione-dependent formaldehyde dehydrogenase in the methylotrophic yeast *Candida boidinii*. Microbiology **148:**2697–2704.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

19. **Marx, C. J., and M. E. Lidstrom.** 2002. A broad-host-range *cre-lox* system for antibiotic marker recycling in Gram-negative bacteria. BioTechniques **33:** 1062–1067.
20. **Marx, C. J., L. Chistoserdova, and M. E. Lidstrom.** 2003. Formaldehyde-detoxifying role of the tetrahydromethanopterin-linked pathway in *Methylobacterium extorquens* AM1. J. Bacteriol. **185:**7160–7168.
21. **Marx, C. J., B. N. O'Brien, J. Breezee, and M. E. Lidstrom.** 2003. Novel methylotrophy genes of *Methylobacterium extorquens* AM1 identified by using transposon mutagenesis including a putative dihydromethanopterin reductase. J. Bacteriol. **185:**669–673.
22. **Palacios, S., V. J. Starai, and J. C. Escalante-Semerena.** 2003. Propionyl coenzyme A is a common intermediate in the 1,2-propanediol and propionate catabolic pathways needed for expression of the *prpBCDE* operon during growth of *Salmonella enterica* on 1,2-propanediol. J. Bacteriol. **185:** 2802–2810.
23. **Pomper, B. K., J. A. Vorholt, L. Chistoserdova, M. E. Lidstrom, and R. K. Thauer.** 1999. A methenyl tetrahydromethanopterin cyclohydrolase and a methenyl tetrahydrofolate cyclohydrolase in *Methylobacterium extorquens* AM1. Eur. J. Biochem. **261:**475–480.
24. **Pomper, B. K., O. Saurel, A. Milon, and J. A. Vorholt.** 2002. Generation of formate by the formyltransferase/hydrolase complex (Fhc) from *Methylobacterium extorquens* AM1. FEBS Lett. **17:**133–137.
25. **Ras, J., P. W. Van Ophem, W. N. Reijnders, R. J. Van Spanning, J. A Duine, A. H. Stouthamer, and N. Harms.** 1995. Isolation, sequencing, and mutagenesis of the gene encoding NAD- and glutathione-dependent formaldehyde dehydrogenase (GD-FALDH) from *Paracoccus denitrificans*, in which GD-FALDH is essential for methylotrophic growth. J. Bacteriol. **177:**247–251.
26. **Rizzitello, A. E., J. R. Harper, and T. J. Silhavy.** 2001. Genetic evidence for parallel pathways of chaperone activity in the periplasm of *Escherichia coli*. J. Bacteriol. **183:**6794–6800.
27. **Sanghani, P. C., H. Robinson, W. F. Bosron, and T. D. Hurley.** 2002. Human glutathione-dependent formaldehyde dehydrogenase. Structures of apo, binary, and inhibitory ternary complexes. Biochemistry **41:**10778–10786.
28. **Simon, R., U. Priefer, and A. Puhler.** 1984. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.
29. **Snel, B., P. Bork, and M. A. Huynen.** 1999. Genome phylogeny based on gene content. Nat. Genet. **21:**108–110.
30. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–964.
31. **Tanaka, N., Y. Kusakabe, K. Ito, T. Yoshimoto, and K. T. Nakamura.** 2002. Crystal structure of formaldehyde dehydrogenase from *Pseudomonas putida*: the structural origin of the tightly bound cofactor in nicotinoprotein dehydrogenases. J. Mol. Biol. **324:**519–533.
32. **Vorholt, J. A., L. Chistoserdova, M. E. Lidstrom, and R. K. Thauer.** 1998. The NADP-dependent methylene tetrahydromethanopterin dehydrogenase in *Methylobacterium extorquens* AM1. J. Bacteriol. **180:**5351–5356.
33. **Vorholt, J. A., L. Chistoserdova, S. M. Stolyar, R. K. Thauer, and M. E. Lidstrom.** 1999. Distribution of tetrahydromethanopterin-dependent enzymes in methylotrophic bacteria and phylogeny of methenyl tetrahydromethanopterin cyclohydrolases. J. Bacteriol. **181:**5750–5757.
34. **Vorholt, J. A., C. J. Marx, M. E. Lidstrom, and R. K. Thauer.** 2000. Novel formaldehyde-activating enzyme in *Methylobacterium extorquens* AM1 required for growth on methanol. J. Bacteriol. **182:**6645–6650.
35. **Wagner, A.** 2000. Robustness against mutations in genetic networks of yeast. Nat. Genet. **24:**355–361.
36. **Whitaker, J. R., and P. E. Granum.** 1980. An absolute method for protein determination based on the difference in absorbance at 235 and 280 nm. Anal. Biochem. **109:**156–159.
37. **Yasueda, H., Y. Kawahara, and S. Sugimoto.** 1999. *Bacillus subtilis yckG* and *yckF* encode two key enzymes of the ribulose monophosphate pathway used by methylotrophs, and *yckH* is required for their expression. J. Bacteriol. **181:**7154–7160.