Identification and characterization of the furfural and 5-(hydroxymethyl)furfural degradation pathways of *Cupriavidus basilensis* HMF14

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The toxic fermentation inhibitors in lignocellulosic hydrolysates pose significant problems for the production of second-generation biofuels and biochemicals. Among these inhibitors, 5-(hydroxymethyl)furfural (HMF) and furfural are specifically notorious. In this study, we describe the complete molecular identification and characterization of the pathway by which *Cupriavidus basilensis* HMF14 metabolizes HMF and furfural. The identification of this pathway enabled the construction of an HMF and furfural-metabolizing *Pseudomonas putida*. The genetic information obtained furthermore enabled us to predict the HMF and furfural degrading capabilities of sequenced bacterial species that had not previously been connected to furanic aldehyde metabolism. These results pave the way for in situ detoxification of lignocellulosic hydrolysates, which is a major step toward improved efficiency of utilization of lignocellulosic feedstock.

hydroxymethyl furfural | degradation | inhibitors | lignocellulosic | hydolysate

Lignocellulosic biomass from grasses, wood, agricultural crop residues, and municipal waste provides an abundant and renewable source of sugars for the fermentative production of fuels and base chemicals. Fermentable sugars may be released from lignocellulosic biomass by a diversity of pretreatment and hydrolysis procedures. A major drawback, however, is the formation of toxic by-products such as organic acids, phenolic compounds, and furan derivatives.

Furfural (2-furaldehyde, CAS number 98-01-1) and 5-(hydroxymethyl)furfural (HMF, CAS number 67-47-0) are key toxic furan derivatives in acid–pretreated lignocellulose hydrolysates (1, 2). These furanic aldehydes cause detrimental effects that are mostly ill-understood at the molecular level, but result in a decrease of specific growth rates, ethanol yields, and productivities in both yeasts and bacteria (for review, see refs. 1, 3, and 4). Efficient detoxification methods are therefore required to improve the utility of lignocellulosic hydrolysate as a fermentation feedstock (1, 5, 6).

Several methods for the removal of furanic compounds have been reported, such as ether extraction, alkaline precipitation, or enzymatically using laccases (for reviews, see refs. 3 and 7). Bioabatement with microorganisms that degrade furan derivatives may present an important alternative approach (8–12). A number of microorganisms that metabolize furfural or HMF have been described (9, 10, 13, 14). A furfural degradation pathway has been proposed based on enzyme activities in *Pseudomonas putida* strains Fu1 (14) and F2 (15). No reports are available on enzymes involved in HMF degradation, and no pathway for the degradation of HMF has been proposed to date. Furthermore, no genetic information on degradation of furanic compounds is available, except for a number of regulatory and accessory genes that were recently reported (16).

Recently, we isolated the previously undescribed HMF and furfural-metabolizing Gram-negative bacterium *Cupriavidus basilensis* HMF14 from soil, by means of enrichment cultures with HMF as the sole carbon source (12). In the present study, we have characterized the HMF and furfural degradation pathways of this bacterium both at the biochemical and the genetic level. The structural genes were expressed in a heterologous host, *Pseudomonas putida* S12, yielding a strain capable of utilizing HMF and furfural as sole carbon sources. Using the newly characterized gene sequences, the furfural or HMF degrading capabilities of other bacteria could be predicted. The previously undescribed insights into the furfural and HMF catabolism of *C. basilensis* HMF14 and other bacteria may be applied to modify fermentation hosts to remove furanic aldehydes in situ. This approach bypasses the requirement for a detoxification pretreatment and improves the amount of total utilizable carbon in lignocellulosic hydrolysate. Thus, unique opportunities are created for the application of this renewable feedstock for the biotechnological production of chemicals and fuels.

Results

Identification of Genes Involved in Furfural and HMF Degradation by Transposon Mutant Screening. A transposon mutant library of *C. basilensis* HMF14 was screened for clones that were unable to grow on furfural and/or HMF. Twenty-five transposon mutants were selected from 14.000 clones, and the chromosomal DNA flanking the transposon insertion sites was sequenced to identify the interrupted genes. Several individual mutants were found to have a transposon inserted in the same gene, underpinning that these genes were essential for furfural and HMF metabolism. Additional primer walking sequencing of up- and downstream regions of these genes revealed two distinct gene clusters, both preceded by a LysR-type transcriptional regulator in the reverse orientation. The nucleotide sequences of these clusters were assigned GenBank accession numbers GU556182 and GU556183. The first cluster contained five genes, designated *hmfABCDE*, whereas the other cluster contained four genes: *hmfFGH'H* (Fig. 1*A*). Insertion of a transposon in either of the two clusters corresponded to two distinct phenotypes. If the *hmfABCDE* cluster was interrupted, no growth occurred on either HMF or furfural, suggesting a—at least partly—shared metabolic pathway for utilization of furfural and HMF. An insertion in the

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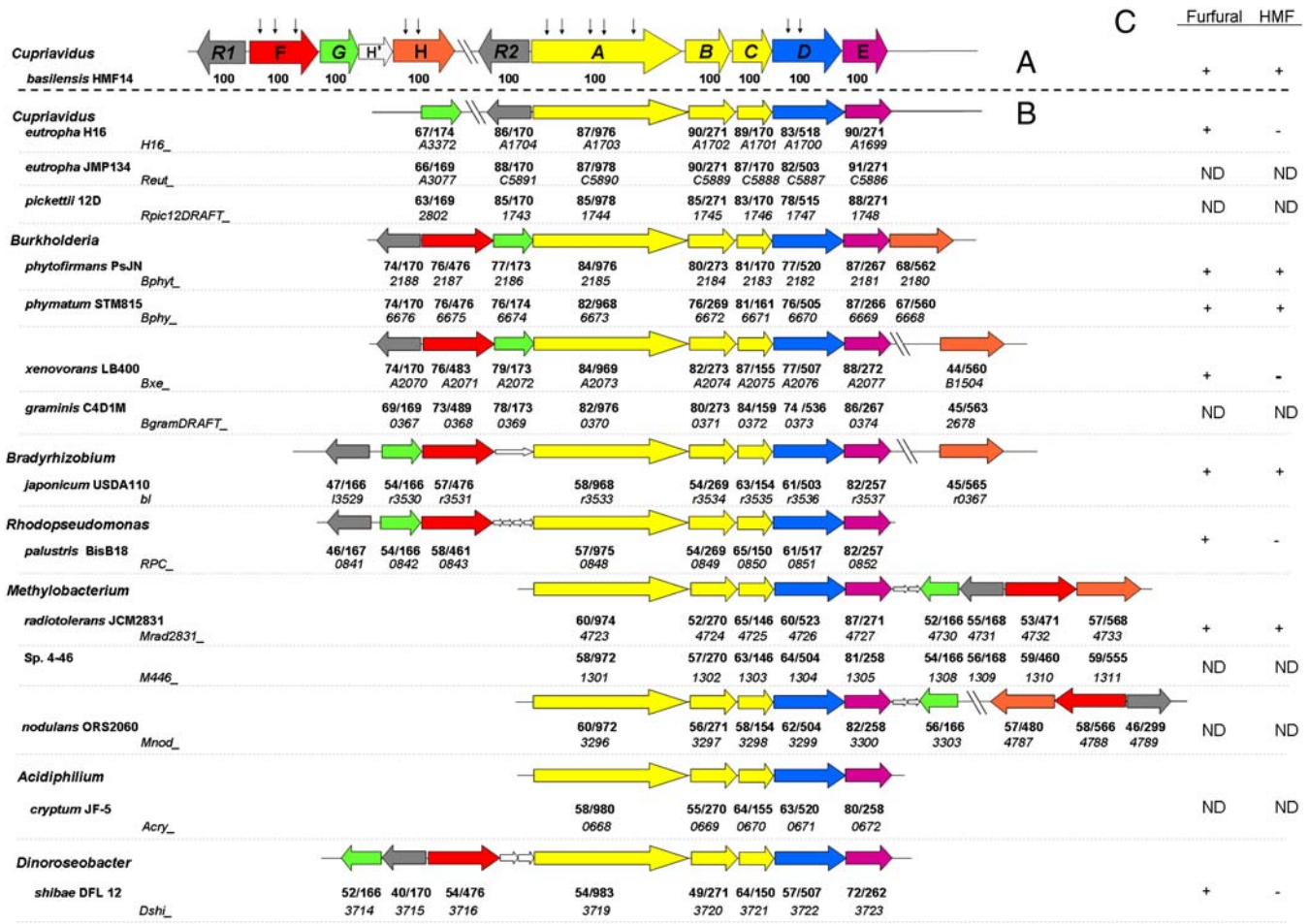

Fig. 1. Schematic representation of the genetic organization of the *hmf* genes for furfural and HMF metabolism in *C. basilensis* HMF14 (*A*) and other species (*B*) that were identified as potential furfural and/or HMF utilizers. Colors correspond to enzyme activities in Fig. 2. Bold numbers (**x**/**y**) below arrows indicate the percentage identity (**x**) to the corresponding *C. basilensis* HMF14 protein in a **y** amino-acid stretch. Orthologous genes were identified by BLASTx homology searches in the nonredundant protein database of the National Center for Biotechnology Information. Hits for the furfural cluster were defined as relevant when orthologues for *hmfA*, *B*, *C*, *D*, and *E* were present in a single genome, with the *hmfA* orthologue encoding an enzyme that was at least 50% identical to HmfA. The same criterion was used to define *hmfF* and *hmfG* orthologues, whereas 40% identity to HmfH was used as the criterion for *hmfH* orthologues. Numbers in italics indicate genome locus tags of the indicated strain. White arrows depict genes with no assigned metabolic function. (*C*) Overview of growth phenotype of tested strains on mineral salts medium with either furfural or HMF (3 mM) as the sole carbon source. ND: not determined.

hmfFGH'H cluster resulted in loss of growth on HMF only. Mutant phenotypes of transposon mutants and BLASTx analysis (17) of the genes included in the two clusters are summarized in Table 1.

Elucidation of the Furfural Catabolic Pathway of *C. basilensis* HMF14. The putative enzyme functions encoded by the *hmfABCDE* cluster of *C. basilensis* HMF14 were in good agreement with the enzyme activities that were reported to constitute the furoic-acid degradation pathway of *Pseudomonas putida* strains F2 and Fu1 (14, 15) (Fig. 2*B*). The first step of this proposed pathway involves an acyl-CoA synthetase to produce 2-furoyl-CoA from 2-furoic acid, which activity matches the putative function of HmfD. The putative function of HmfD was supported by the accumulation of 2-furoic acid in *hmfD*-disrupted transposon mutants of *C. basilensis* HMF14 when cultured in the presence of furfuryl alcohol or furfural. Furthermore, it was established that 2-furoic acid is the substrate for ATP-dependent CoA ligation by HmfD. This activity was present in cell extracts of wild-type *C. basilensis* HMF14 (316 ± 26.1 U $\cdot$ g^{-1}) and *P. putida* S12 expressing HmfD (345 ± 24.5 U $\cdot$ g^{-1}), whereas it was absent in *C. basilensis* HMF14 transposon mutants in which *hmfD* was disrupted.

In *P. putida* F2 and Fu1, 2-furoyl-CoA is converted into 5-hydroxy-2-furoyl-CoA by a molybdenum-dependent 2-furoyl-CoA dehydrogenase. The proteins encoded by *hmfABC* in *C. basilensis* HMF14 correspond to the three subunits that constitute bacterial Mo-dependent dehydrogenases. Functionality of *hmfABC* was confirmed by demonstrating furoic-acid dependent Nitro Blue Tetrazolium reducing activity in cell extracts of *C. basilensis* HMF14 (21 ± 5.7 U $\cdot$ g^{-1}) and *P. putida* S12 coexpressing HmfABC and HmfD (42 ± 4 U $\cdot$ g^{-1}). The latter activity was required to generate 2-furoyl-CoA from 2-furoic acid as the substrate for HmfABC (Fig. 2*A*).

In the final steps of the proposed furoic-acid metabolic pathway of *P. putida* strains Fu1 and F2, 5-hydroxy-2-furoyl-CoA is converted into 2-oxoglutarate and CoA via a combination of spontaneous keto-enol tautomerizations, delactonization and thioester hydrolysis (Fig. 2*B*). No enzyme activities had been previously specified for the lactone and thioester hydrolysis, and no clear function could be assigned to the remaining gene of the *hmfABCDE* cluster, *hmfE*. Closest HmfE-homologues were annotated as proteins of the enoyl-CoA hydratase/isomerase family. This family encompasses a wide variety of enzymes with diverse activities and also includes enoyl-CoA hydrolases (18, 19). This may suggest a role of HmfE in CoA-thioester hydrolysis, which was tested by incubating cell suspensions of *P. putida*

Table 1. Growth phenotype of selected *C. basilensis* HMF14 transposon mutants, and BLASTx analysis and assigned function of genes involved in furfural and HMF degradation

Gene	Growth phenotype of transposon mutant			Best BLASTx hit (Acc. No)	Assigned function
	MM + citrate	MM + furfural	MM + HMF		
hmfA	+	–	–	Aerobic-type carbon monoxide dehydrogenase homologue, subunits L and G (YP_726196)	Furoyl-CoA dehydrogenase large subunit
hmfB *				Carbon-monoxide dehydrogenase (YP_293089)	Furoyl-CoA dehydrogenase FAD binding subunit
hmfC *				Aerobic-type carbon monoxide dehydrogenase 2Fe-2S iron-sulfur subunit (YP_726194)	Furoyl-CoA dehydrogenase 2Fe-2S iron sulfer subunit
hmfD	+	–	–	Acyl-CoA synthetase (YP_726193)	Furoyl-CoA syntethase
hmfE *				Enoyl-CoA hydratase/isomerase (YP_293086)	2-oxoglutaroyl-CoA hydrolase
hmfF	+	+	–	UbiD family decarboxylase (YP_001895811)	2,5-furan-dicarboxylic acid decarboxylase 1
hmfG	+	+	–	3-octaprenyl-4-hydroxybenzoate carboxy-lyase (ZP_02881560)	2,5-furan-dicarboxylic acid decarboxylase 2
hmfH' *				hypothetical protein(YP_293096)	NA
hmfH	+	+	–	Glucose-methanol-choline oxidoreductase (YP_001895804)	HMF/furfural oxidoreductase
hmfR1 *				LysR family transcriptional regulator (YP_001862747.1)	Putative LysR-type transcriptional regulator
hmfR2 *				LysR family transcriptional regulator (YP_293091.1)	Putative LysR-type transcriptional regulator

NA, no assigned function.
*The mutant phenotype was not determined since no transposon mutant was available.

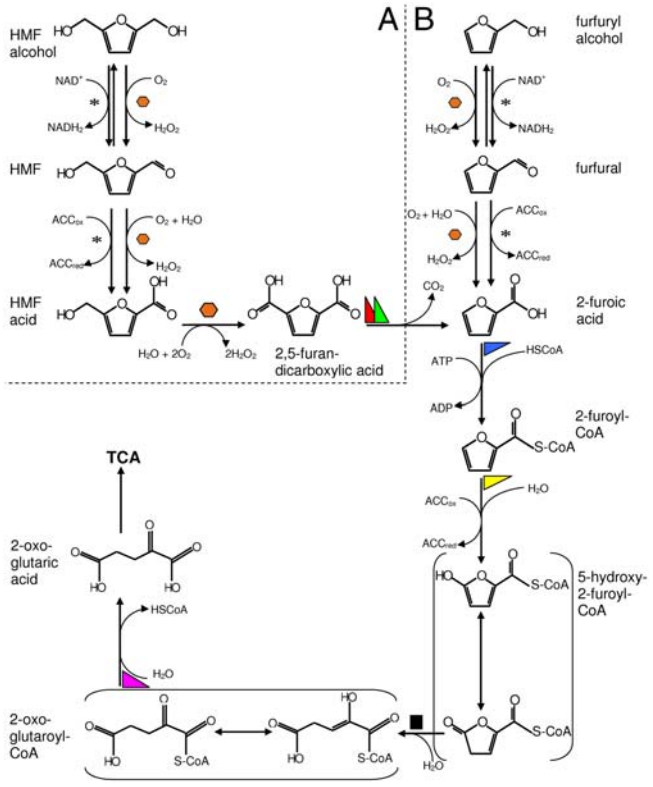

Fig. 2. Graphical representation of the HMF (*A*) and furfural (*B*) metabolic pathway in *C. basilensis* HMF14. *B* has been adapted from Koenig and Andreesen (14). Colored hexamers and triangles indicate enzymes with the following activities: orange hexagon, furfural/HMF oxidoreductase; red and green triangles, 2,5-furan-dicarboxylic acid decarboxylase; blue triangle, 2-furoyl-CoA synthetase; yellow triangle, furoyl-CoA dehydrogenase; purple triangle, 2-oxoglutaryl-CoA hydrolase. Colors correspond to the genes depicted in Fig. 1*A*. ■; indicates a lactone hydrolysis that may occur spontaneously, or may be catalyzed by a generic lactone hydrolase. Double-pointed arrows indicate keto-enol tautomerizations. Reactions marked with (*) can be catalyzed either by HmfH or by (probably nonspecific) dehydrogenases. ACC; acceptor, which is oxidized$_{(ox)}$ or reduced$_{(red)}$.

S12 expressing HmfABCDE with 10 mM furoic-acid. Arsenite (1 mM) was added to inhibit 2-oxoglutarate dehydrogenase. After overnight incubation 3 mM of 2-oxoglutarate had accumulated, which is in agreement with previous experiments performed on *P. putida* Fu1 (14). No 2-oxoglutarate was formed with control cells of *P. putida* S12 expressing HmfABCD. Based on these findings, it was concluded that *hmfE* encodes a specific 2-oxoglutaroyl-CoA thioesterase. This part of the pathway shows strong similarity to the 3-methylmuconolactone degradation route described by Cha et al. (20), in which the CoA-thioester remains attached after delactonization. The lactone-CoA intermediate may hydrolyze spontaneously, or lactone hydrolysis is catalyzed by a nonspecific hydrolase.

In the furfural cluster, no genes were identified for the pathway upstream of furoic acid, i.e., the oxidations of furfuryl alcohol to furfural and furoic acid (Fig. 2). Furfuryl alcohol was found to be oxidized to furfural by an NAD-dependent dehydrogenase activity. The oxidation of furfural to furoic acid was catalyzed by a dehydrogenase activity with unknown cofactor that could be replaced by phenazine methosulphate/dichlorophenolindophenol in vitro. Such activities were observed both in *C. basilensis* HMF14 and *P. putida* S12 (Table 2). Therefore, and since none of the selected *C. basilensis* HMF14 transposon mutants were found to be interrupted in genes encoding alcohol or aldehyde dehydrogenases, it was presumed that redundant, nonspecific dehydrogenases were responsible for the upper pathway oxidation reactions.

Elucidation of the HMF Catabolic Pathway of *C. basilensis* HMF14. The HMF degradation pathway of *C. basilensis* HMF14 was reconstructed based on putative gene functions of the *hmfFGH'H* cluster. The *hmfFG* genes encode two putative decarboxylases of the UbiD/UbiX type that commonly operate in concert (21, 22). *C. basilensis* HMF14 mutants with disrupted *hmfFG* genes accumulated HMF acid and 2,5-furandicarboxylic acid (FDCA) when cultured in the presence of HMF, which suggested that these carboxylic acids were the substrate for HmfFG. Cell extracts of both wild-type *C. basilensis* HMF14 and *P. putida* S12 expressing HmfFG formed 2-furoic acid when incubated with FDCA (6.0 ± 0.1 U $\cdot$ g^{-1} and 8.6 ± 0.7 U $\cdot$ g^{-1}, respectively). HMF acid was not decarboxylated to furfuryl alcohol, demonstrating that

Table 2. Dehydrogenase activities measured in cell extract of wild-type *C. basilensis* HMF14 and *P. putida* S12

Enzyme activity	Substrate	Product	Cofactor	*C. basilensis* HMF14	*P. putida* S12
Aldehyde reductase	Furfural	Furfuryl alcohol	NADH	109 ± 81	3305 ± 685.4
Alcohol dehydrogenase	Furfuryl alcohol	Furfural	NAD^+	28 ± 10.4	172 ± 13.1
Aldehyde dehydrogenase	Furfural	Furoic acid	PMS/DCPIP	245 ± 25	410 ± 169.5
Aldehyde reductase	HMF	HMF alcohol	NADH	58 ± 7.5	925 ± 268.9
Alcohol dehydrogenase	HMF alcohol	HMF	NAD^+	ND	ND
Aldehyde dehydrogenase	HMF	HMF acid	PMS/DCPIP	12 ± 1.5	27 ± 12.8

ND, not determined for lack of commercially available substrate. Activities depicted in U g^{-1} protein (1 U represents the amount of protein that converts 1 μmol of substrate per min). PMS/DCPIP: phenazine methosulphate/2,6-dichlorophenol-indophenol. Errors denote the deviation of the mean.

FDCA was the actual substrate for HmfFG. Thus, HMF degradation in *C. basilensis* HMF14 proceeds obligately via its dicarboxylic acid form. No decarboxylase activity was observed in a cell extract of *P. putida* S12 expressing HmfG only. When HmfF was expressed as a single enzyme only slight decarboxylase activity was observed (0.7 ± 0.1 U $\cdot$ g^{-1}), demonstrating that both proteins are required for optimal FDCA decarboxylase activity.

The *hmfH* gene encodes a putative FAD-dependent oxidoreductase. *C. basilensis* HMF14 mutants with a disrupted *hmfH* gene accumulated HMF acid when cultured in the presence of HMF. Cell extracts of both wild-type *C. basilensis* HMF14 and *P. putida* S12 expressing HmfH formed FDCA when incubated with HMF acid, confirming that HmfH catalyzes the oxidation of the HMF-monocarboxylic acid to the dicarboxylic acid form. No FDCA was formed when oxygen was removed, demonstrating that HmfH is a true oxidase.

The *hmfH'* gene encodes a hypothetical protein with 49% identity over a stretch of 296 amino acids to a probable extracytoplasmic solute receptor of *Ralstonia eutropha* H16. This gene may play a role in HMF transport, but a metabolic function was considered unlikely (23).

Analogous to the furfural pathway, no specific genes encoding dehydrogenases were identified for the oxidations in the upper HMF metabolic pathway leading from HMF alcohol to HMF and HMF acid. Also these oxidations were concluded to be performed by nonspecific, redundant dehydrogenases whose activities were observed both in *C. basilensis* HMF14 and *P. putida* S12 (Table 2). However, it was observed that HmfH could also oxidize HMF, furfural, and furfuryl alcohol to the corresponding acids. Apparently, this oxidase is essential for the formation of FDCA from HMF acid but also provides an oxidase alternative to the nonspecific alcohol and aldehyde dehydrogenases that constitute the upper metabolic pathways for HMF and furfural.

Based on the above observations, the pathway depicted in Fig. 2*A* was constructed for HMF catabolism. First, HMF is oxidized to HMF acid, either by nonspecific dehydrogenases or by HmfH. Subsequently, HMF acid is oxidized to FDCA for which conversion HmfH is essential. The HMF and the furfural catabolic pathways converge at the level of 2-furoic acid upon decarboxylation of FDCA by HmfFG.

Heterologous Expression of the Furfural and HMF Degradation Pathways in *P. putida* S12. Functional characterization of the furfural and HMF catabolic genes of *C. basilensis* HMF14 enabled a reconstruction of the complete catabolic pathway for these furanic compounds. For a final verification of the functionality of the reconstructed pathway, the encoding genes were expressed in a heterologous host, *P. putida* S12.

First, the furfural cluster *hmfABCDE* was introduced into *P. putida* S12. As expected, the resulting strain, *P. putida* S12 pJT′hmfABCDE, was able to utilize furoic acid, furfural, and furfuryl alcohol as sole carbon sources, although growth was initially poor. Therefore, strain S12 pJT′hmfABCDE was repeatedly transferred to a fresh mineral salts medium with furfural as the sole carbon source. After 10 serial transfers, *P. putida* strain S12_fur was obtained, which showed a reproducible growth rate of 0.3 h^{-1} on furfural as a sole carbon source with a biomass yield of 51% (C-mol biomass/C-mol substrate). Wild-type *P. putida* S12 and the *P. putida* S12 strains expressing only HmfABCD or HmfABC both failed to grow on furoic acid, even after prolonged incubation. These results confirmed that all genes required for furfural metabolism are located in the furfural cluster *hmfABCDE* and that all genes in this cluster are essential for furfural metabolism, including the *hmfE*-encoded CoA-thioester hydrolase.

Subsequently, the *hmfFGH* genes were cloned into *P. putida* S12_fur. The resulting strain, *P. putida* S12_HMF, utilized either furfural or HMF as the sole carbon source, at a growth rate of 0.23 h^{-1} and a yield of 40% (C-mol biomass/C-mol substrate). This growth rate was comparable with the growth rate of *C. basilensis* HMF14 on furfural and HMF (0.22 and 0.25 h^{-1}, respectively). Without the addition of the *hmfFGH* genes, no growth was observed on HMF by *P. putida* S12_fur. Gene *hmfH'* was apparently dispensable for growth on HMF, confirming that the encoded protein has no essential function in HMF metabolism. Nor was the gene essential for HMF transport in *P. putida* S12. Thus, also all genes required for the utilization of HMF were characterized, and their functionality was reconfirmed by functional expression in a heterologous host.

Identification of Other Bacteria Capable of Degrading Furfural and HMF. The sequence of the *C. basilensis* HMF14 furfural and HMF catabolic genes were used in BLAST searches for similar sequences in publicly available microbial genomes. Remarkably, orthologous genes were found in a relatively limited group of Gram-negative bacteria belonging to the genera *Cupriavidus*, *Burkholderia*, *Bradyrhizobium*, *Rhodopseudomonas*, *Acidiphilium*, *Dinoroseobacter*, and *Methylobacterium* (24–30) (Fig. 1). Eight of these potential furfural or HMF degrading bacteria were selected and tested for the ability to utilize furfural or HMF as the sole carbon source (Fig. 1*C*).

The organization of the genes in the *hmfABCDE* ("furfural") cluster was identical in all species identified by BLAST searches, indicating that this cluster is highly conserved. Indeed, all tested bacteria possessing *hmfABCDE* orthologues utilized furfural as a sole carbon source (Fig. 1*C*). The organization of *hmfF*, *hmfG*, and *hmfH*, on the other hand, was highly diverse. Of these genes, the *hmfH* orthologues appeared to be the least conserved, encoding oxidases that are between 68 and 43% identical to HmfH. Although the presence of *hmfFGH* orthologues correlated well with the ability to utilize HMF (Fig. 1*C*), the percentage identity of the ill-conserved oxidase to HmfH appeared to be crucial. *Bradyrhizobium japonicum* USDA110 utilized HMF, having an oxidase that was 45% identical to HmfH. By contrast, *Burkholderia xenovorans* LB400 was unable to utilize HMF although its oxidase was 44% identical to HmfH. This observation suggests that the relative high similarity to HmfH may point at HMF-acid oxidative function. Keeping this in mind, the capability to utilize furfural and/or HMF could be well predicted in other

bacteria based on the presence of furfural and HMF metabolic gene clusters.

Discussion

In this paper the HMF and furfural metabolic pathways of *C. basilensis* HMF14 were identified, and the genes involved were isolated and characterized. Previous to the present study only fragmented knowledge was available on enzymes involved in furanic aldehyde metabolism (13–16), while the genetic background on the metabolism of these compounds was not known to date. Such knowledge is extremely valuable in alleviating furanic aldehyde inhibition, which is a serious problem in fermentative production of biofuels and (base) chemicals from acid-pretreated lignocellulosic hydrolysate feedstock.

With respect to furfural metabolism, our findings partially confirmed a putative route previously proposed for *P. putida* strains Fu1 and F2 (14, 15). In addition, a unique enzyme essential for furfural degradation was identified. This enzyme, encoded by *hmfE*, is likely a 2-oxoglutaroyl-CoA-thioester hydrolase, although it could not be excluded that CoA-thioester hydrolysis occurs prior to the lactone hydrolysis as suggested previously (15). No specific lactone hydrolases were found to be essential for furfural metabolism, suggesting that lactone hydrolysis either occurs spontaneously or is catalyzed by a nonspecific lactonase.

We have identified and characterized a previously undescribed pathway for the utilization of HMF. It was demonstrated that the degradation of HMF proceeds via FDCA. This compound is decarboxylated to furoic acid, which is further metabolized by the furfural degradation route. The formation of FDCA from HMF requires an FAD-dependent oxidoreductase, encoded by *hmfH* that shows a mere 68% identity to the closest homologue in the nonredundant Genbank database. This unique oxidoreductase was found to be essential for the oxidation of HMF acid to FDCA, but it can also oxidize HMF alcohol, HMF, furfuryl alcohol, and furfural to the corresponding monocarboxylic acid forms. Thus, HmfH provides an alternative to the nonspecific dehydrogenases that also perform these upper pathway oxidations in *C. basilensis* HMF14. Such nonspecific "furanic dehydrogenases" were also observed in *P. putida* S12, as well as in other microorganisms that cannot utilize furanic aldehydes for growth, probably serving to detoxify furanic aldehydes (31–33).

In addition to the genes encoding the core enzymes of the furanic aldehyde metabolic pathways, several other genes were identified in the transposon mutant library screening, or were present adjacent to the HMF and furfural gene clusters (*SI Text*). These genes apparently had an indirect relation with furanic aldehyde metabolism, encoding proteins with diverse functions that are putatively involved in transcriptional regulation, transport, stress tolerance, and cofactor metabolism. Homologues of some of these genes were recently isolated from *P. putida* Fu1 as genes relating to furfural degradation or toxicity (16). The identified *P. putida* Fu1 genes also included a *lysR*-type regulator gene that was essential for furoic-acid metabolism. This protein encoding this gene shows 43% identity over a 298 amino acid stretch to *hmfR1* and 47% identity over a 171 amino acid stretch to *hmfR2*, which supports their putative function as transcriptional regulators of the HMF and furfural clusters of *C. basilensis* HMF14.

The characterization of the biochemical degradation routes of HMF and furfural paves the way for constructing industrial production hosts that remove these toxicants from lignocellulosic hydrolysate in situ. Thus, detoxification pretreatments prior to fermentation may be omitted, simplifying process setup and contributing to cost-effectiveness of lignocellulose-based fermentation processes. As demonstrated in the present study, the HMF and furfural metabolic pathway can be functionally expressed in *P. putida*, but the molybdenum-dependent furoyl-CoA dehydrogenase encoded by *hmfABC* puts a constraint on the host with regard to the requirement for a molybdenum cofactor (MoCo) biosynthetic pathway. The same holds for the oxygen-dependent HmfH, which currently limits the applicability of the HMF degradation pathway to aerobic hosts. The applicability of the furfural and HMF pathway could be broadened by employing an alternative, non-O_2-dependent HMF/furfural oxidizing enzyme and a non-MoCo furoyl-CoA dehydrogenase; this approach is currently pursued in our laboratory. The identification of the genes responsible for HMF and furfural degradation furthermore enables the identification of other bacteria capable of degrading furfural and HMF. Thus, more unique pathways for furfural and HMF metabolism may be expected to emerge from database mining in the near future. This will extend the possibilities of implementing these pathways in industrial fermentation hosts to overcome the furanic aldehyde inhibition connected with the use of lignocellulosic hydrolysate as renewable fermentation feedstock.

Materials and Methods

Strains and Culture Conditions. Strains and plasmids used in this study and the culture conditions are presented in *SI Text*. Carbon sources were added to the mineral salts media (mineral salts medium (MM), adapted from ref. 34) as indicated. Antibiotics were added as required, in the following concentrations: ampicillin (amp), 100 μg/ml (*Escherichia coli*); gentamicin, 30 μg/ml in Luria broth (LB), 10 μg/ml in MM (*P. putida* S12 (35)); kanamycin, 50 μg/ml and tetracycline, 10 mg/L (*E. coli*), and 15 mg/L (*C. basilensis* HMF14 (12)). *E. coli* was cultured at 37 °C; all other bacteria were routinely cultured at 30 °C. Shake flask cultures in mineral salts media were performed in amber Boston bottles in a horizontally shaking incubator. Cultures in LB were performed in Erlenmeyer flasks.

Transposon Mutagenesis and Mutant Library Screening. A transposon mutant library of *C. basilensis* HMF14 was constructed using a modified version of plasposon pTnModKmO (36), in which the kanamycin marker gene (36, 37) was replaced with a *tetA* tetracycline marker flanked by *loxP* sites (*SI Text*) (38, 39). The resulting plasposon pTnModTcO(lox) was introduced in *C. basilensis* HMF14 by triparental mating using *Escherichia coli* pRK2013 as the mobilizing strain (37, 40). *C. basilensis* HMF14 transposon mutants were selected on *Pseudomonas* isolation agar (Difco) supplemented with 60 mg/L Tc and transferred to 96-well plates. The mutant library was screened on solid MM supplemented with 15 mg/l Tc and 10 mM of either furfural, HMF or citrate (positive control substrate). Citrate$^+$, furfural$^-$ and/or HMF$^-$ colonies were selected for further study after pure culturing and reconfirmation of the phenotype.

DNA Techniques. All primers used are displayed in *SI Text*, and the construction of expression plasmids is described in *SI Text*. Maps of the expression plasmids pJT′mcs and pBT′mcs are presented in *SI Text*. Genomic DNA, plasmid DNA, and agarose-trapped DNA fragments were isolated with commercial kits (QIAGEN). PCR reactions were performed with Accuprime *Pfx* polymerase (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was introduced into electrocompetent cells using a Gene Pulser electroporation device (BioRad). Chromosomal DNA flanking transposon insertion sites were isolated by standard methods (41). Oligonucleotide synthesis and nucleotide sequencing were performed by Eurofins MWG operon.

Analytical Methods. Cell dry weight (CDW) content of bacterial cultures was determined by measuring optical density at 600 nm (OD_{600}) using a conversion factor per OD_{600} unit of 0.56 g CDW/L [Biowave Cell Density Meter (WPA Ltd)] or 1.4 g CDW/L [μQuant MQX200 microplate reader (Biotek) using flat-bottom 96-well microplates (Greiner)].

HPLC analyses were performed on an Agilent 1100 system equipped with a diode array detector. Furanic compounds were measured as previously described (12). Using an Aminex HDP-87H column (BioRad, 300 × 7.8 mm), 2-Oxoglutarate was detected at 210 nm. As eluent, 4 mM H_2SO_4 was used at a flow of 0.6 ml/min. Alternatively, 2-oxoglutarate was measured in an enzymatic assay using glutamate dehydrogenase as described by Trudgill et al. (15). Protein concentrations were measured using Bradford reagent (Sigma-Aldrich).

Chemicals. FDCA was purchased from Immunosource B.V. 5-Hydroxymethylfuroic acid (HMF acid) was purchased from Matrix Scientific. This compound was found to be highly esterified. Immediately prior to use 14.6 mg of HMF acid was dissolved in 10 mL of demineralized water and boiled for 2 h in 2 M H_2SO_4, cooled, and adjusted to pH 7.0 with NaOH after addition of 50 mM of

phosphate buffer. This solution was assumed to be 7.5 mM stock, after confirmation of deesterification by HPLC analysis. All other chemicals were purchased from Sigma-Aldrich Chemie B.V.

Enzyme Assays. Enzyme activities of the furanic aldehyde pathways were measured in cell extracts of wild-type *C. basilensis* HMF14 or *P. putida* S12 transformants expressing the proper enzyme(s). As a negative control, wild-type *P. putida* S12 was used, or a *C. basilensis* HMF14 transposon mutant with the appropriate gene disrupted. Cell extracts were prepared by sonication from 15-fold concentrated late-log phase cultures on MM supplemented with 12 mM succinic acid. After removing cell debris, the supernatant was desalted using a PD10 gel filtration column (GE Healthcare). Enzyme activities of the furfural metabolic pathway were assessed by methods adapted from previous reports (13,14) (*SI Text*).

HMF/furfural oxidoreductase activity was determined by incubating cell extract with furfural, furfuryl alcohol, HMF, or HMF-acid at 30 °C under oxygenated conditions. The reaction mixture contained 1.38 mL cell extract, 0.4 mL oxygen-saturated MM, and 20 μl of 2 mM flavin-adenine dinucleotide (FAD). The reaction was started by addition of 0.2 mL of a 10 mM substrate stock solution (furfural, furfuryl alcohol, or HMF). Samples were drawn at set intervals and analyzed by HPLC. Immediately after sampling, the reaction was stopped by addition of HCl to a final concentration of 1 M. Oxygen-depleted controls were incubated in headspace vials with a rubber stopper under nitrogen gas. Prior to starting the reaction, oxygen was stripped from the reaction mixture with a continuous stream of nitrogen gas.

FDCA decarboxylase activity was determined by incubating 1.38 mL cell extract, 0.4 mL MM, and 20 μl of 1 mM pyridoxal 5′-phosphate. The reaction was started by addition of 0.2 mL of 10 mM FDCA. Samples were drawn at set intervals and analyzed by HPLC. Immediately after sampling, the reaction was stopped by addition of HCl to a final concentration of 1 M. One unit is defined as the activity catalyzing the conversion of 1 μmol of furoic acid per min at 30 °C.

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