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Microbial degradation of furanic compounds: biochemistry, genetics, and impact

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Abstract Microbial metabolism of furanic compounds, especially furfural and 5-hydroxymethylfurfural (HMF), is rapidly gaining interest in the scientific community. This interest can largely be attributed to the occurrence of toxic furanic aldehydes in lignocellulosic hydrolysates. However, these compounds are also widespread in nature and in human processed foods, and are produced in industry. Although several microorganisms are known to degrade furanic compounds, the variety of species is limited mostly to Gram-negative aerobic bacteria, with a few notable exceptions. Furanic aldehydes are highly toxic to microorganisms, which have evolved a wide variety of defense mechanisms, such as the oxidation and/or reduction to the furanic alcohol and acid forms. These oxidation/reduction reactions constitute the initial steps of the biological pathways for furfural and HMF degradation. Furfural degradation proceeds via 2-furoic acid, which is metabo-

lized to the primary intermediate 2-oxoglutarate. HMF is converted, via 2,5-furandicarboxylic acid, into 2-furoic acid. The enzymes in these HMF/furfural degradation pathways are encoded by eight *hmf* genes, organized in two distinct clusters in *Cupriavidus basilensis* HMF14. The organization of the five genes of the furfural degradation cluster is highly conserved among microorganisms capable of degrading furfural, while the three genes constituting the initial HMF degradation route are organized in a highly diverse manner. The genetic and biochemical characterization of the microbial metabolism of furanic compounds holds great promises for industrial applications such as the biotransformation of lignocellulosic hydrolysates and the production of value-added compounds such as 2,5-furandicarboxylic acid.

Keywords Furfural · Hydroxymethylfurfural · Lignocellulosic hydrolysate · Metabolic pathway · Detoxification

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Introduction

Due to the indiscriminate use of fossil resources, the need for renewable feedstocks is becoming increasingly evident. Lignocellulosic biomass is a readily available and abundant feedstock, which can be converted into fermentable sugar by means of thermochemical and/or enzymatic treatments (Taherzadeh et al. 1997; Palmqvist and Hahn-Hagerdal 2000a, b; Lin and Tanaka 2006; Abril and Abril 2009; Parawira and Tekere 2011). These lignocellulosic hydrolysates have been heralded as the future feedstock for fermentative production of biobased fuels and chemicals (Ragauskas et al. 2006).

For efficient release of sugars from lignocellulosic material, pretreatment of the raw biomass is essential. The pretreatment makes the biomass accessible to hydrolyzing chemicals or enzymes by breaking up the lignin structure and disrupting the crystalline structure of the cellulose fibers (Taherzadeh and Karimi 2008). Although many pretreatment approaches have been developed over the years, dilute acid hydrolysis appears to be the most preferred technique for large-scale application. This pretreatment is relatively harsh, however, giving rise to the formation of unwanted and often inhibitory byproducts such as furanic aldehydes, weak acids, and phenolic compounds (Palmqvist and Hahn-Hagerdal 2000b; Thomsen et al. 2009; Klinker et al. 2004; Larsson et al. 1999).

Of the inhibitors formed during acid pretreatment, the furanic aldehydes are considered particularly undesirable due to their relative abundance and toxic effect (Heer and Sauer 2008; Zaldivar et al. 1999). These furanic aldehydes inhibit the hydrolytic enzymes used to liberate the sugars from the (hemi-)cellulose fractions (Jing et al. 2009), as well as growth and metabolism of the microorganisms used in the subsequent fermentation process (Klinker et al. 2004; Palmqvist and Hahn-Hagerdal 2000b). Thus, the occurrence of compounds like furfural and 5-hydroxymethylfurfural (HMF; Fig. 1) can considerably decrease the yield and productivity of fermentative processes based on lignocellulosic hydrolysate (for reviews, see Almeida et al. 2009; Palmqvist and Hahn-Hagerdal 2000b; Thomsen et al. 2009).

The formation of inhibitors during biomass (pre-)treatment may be prevented by careful control of the process parameters. Although considerable progress has been made in lab-scale hydrolysis processes (Kumar et al. 2009), it should be noted that the formation of inhibitory by-products is not easily prevented in an economical way at an industrial scale. Hence, it is often preferred to remove inhibitors prior to fermentation. Several approaches have been investigated, ranging from overliming to solvent extraction and biological detoxification (Mussatto and Roberto 2004; Palmqvist and Hahn-Hagerdal 2000a; Nichols et al. 2010). Alternatively, microbial fermentation hosts may be selected or engineered to tolerate or even metabolize the toxic compounds from lignocellulose hydrolysates. This approach has resulted in host organisms that can tolerate higher concentrations of furfural (Pettersson et al. 2006; Heer and Sauer 2008), that metabolize acetate (Medina et al. 2010), furfural, and HMF (Koopman et al. 2010b; Lopez et al. 2004; Table 1), and that efficiently produce polyhydroxyalkanoates from bagasse hydrolysate (Yu and Stahl 2008). In order to introduce or optimize such properties in a targeted manner, detailed insight into inhibitor tolerance and metabolism is the key. The latter has been the subject of intensive study over the last decade. This review aims at providing an overview of

these studies, with a special focus on microbial metabolism of furfural and HMF.

Formation and occurrence of furanic compounds

The recent academic and industrial interest in furfural and HMF can be attributed to a large extent to their occurrence in lignocellulosic hydrolysates. Up to 7.2 g/l of furanic compounds can be found in these hydrolysates, although the exact amount largely depends on the type of lignocellulose used and the pretreatment and hydrolysis process employed (Almeida et al. 2009). However, these compounds can also be detected in other sources. In nature, furfural (derived from Latin *furfur*, bran) is the most abundant of the simple furanic compounds (Dean 1963). Typically, HMF and furfural are associated with sugar-rich or desiccated foods like honey, coffee, and dried fruits (National Toxicology Program 1990, 2010). Through these foods, humans are regularly exposed to furfural and HMF, as is also evident from the common occurrence of furanic compound derivatives such as 2,5-furandicarboxylic acid in human urine (Flaschenträger and Wahhab 1960). Although there is some evidence of carcinogenic effects from high doses of these furanic aldehydes (National Toxicology Program 1990, 2010), HMF is also explored for its beneficial potential in treating human diseases (Lin et al. 2008; Ding et al. 2010; Michail et al. 2007).

At present, it is unclear whether naturally occurring furanic aldehydes emerge purely from abiotic dehydration or that also enzymatic reactions are involved. Furfural and HMF are formed abiotically by threefold dehydration of pentose, respectively, hexose sugars, usually catalyzed by mineral acid (Chheda et al. 2007). Several publications report on HMF and furfural as fungal metabolites (Sumiki 1931; Kawarada et al. 1954). However, it was not unequivocally demonstrated that their formation was biogenic as their presence may also be attributed to the heat sterilization of growth media or the sample extraction procedure (Dean 1963).

In industry, furfural is chemically produced at large scale for application as solvent or as a building block for resins. HMF and furfural are additionally applied as flavor compounds and in the manufacture of pharmaceuticals (Lin et al. 2008; Ding et al. 2010; Michail et al. 2007).

Furanic compound-degrading microorganisms

In 1964, Kakinuma and Yamatodani (1964) were the first to report the isolation of microorganisms capable of degrading furanic compounds “for the purpose of demonstrating the metabolism of furan compounds by microorganisms.”

Table 1 Overview of species capable of degrading furanic compounds

Strain	Year	Substrate	Order ^a	Reference
<i>Amorphotheca resinae</i> ZN1	2010	Furfural and HMF	F Leotiomyces incertae sedis	Zhang et al. (2010)
<i>Cupriavidus basilensis</i> HMF14	2010	Furfural and HMF	– Burkholderiales	Wierckx et al. (2010)
<i>Arthrobacter nicotianae</i>	2010	Furfural and HMF	+ Actinomycetales	Wierckx et al. (2010)
<i>Telluria mixta</i>	2010	Furfural and HMF	– Burkholderiales	Wierckx et al. (2010)
<i>Burkholderia phytofirmans</i> PsJN	2010	Furfural and HMF	– Burkholderiales	Koopman et al. (2010b)
<i>Burkholderial phymatum</i> STM815	2010	Furfural and HMF	– Burkholderiales	Koopman et al. (2010b)
<i>Bradyrhizobium japonicum</i> USDA110	2010	Furfural and HMF	– Rhizobiales	Koopman et al. (2010b)
<i>Rhodopseudomonas palustris</i> BisB18	2010	Furfural	– Rhizobiales	Koopman et al. (2010b)
<i>Methylobacterium radiotolerans</i> JCM2831	2010	Furfural and HMF	– Rhizobiales	Koopman et al. (2010b)
<i>Dinoroseobacter shibae</i> DFL 12	2010	Furfural	– Rhodobacterales	Koopman et al. (2010b)
<i>Ralstonia eutropha</i> H16	2010	Furfural	– Burkholderiales	Koopman et al. (2010b)
<i>Burkholderia xenovorans</i> LB400	2010	Furfural	– Burkholderiales	Koopman et al. (2010b)
<i>Ralstonia eutropha</i>	2008	Furfural	– Burkholderiales	Yu and Stahl (2008)
<i>Pseudomonas putida</i>	2008	Furfural	– Pseudomonadales	Trifonova et al. (2008a)
<i>Serratia plymouthica</i>	2008	Furfural	– Enterobacteriales	Trifonova et al. (2008a)
<i>Rhizobium radiobacter</i>	2008	Furfural	– Rhizobiales	Trifonova et al. (2008a)
<i>Methylobacterium radiotolerans</i>	2008	Furfural	– Rhizobiales	Trifonova et al. (2008a)
<i>Leifsonia xyli</i> ssp. xyli	2008	Furfural	+ Actinomycetales	Trifonova et al. (2008a)
<i>Agromyces aurantiacus</i>	2008	Furfural	+ Actinomycetales	Trifonova et al. (2008a)
<i>Coniochaeta ligniaria</i>	2008	Furfural	F Coniochaetales	Trifonova et al. (2008a)
<i>Coniochaeta ligniaria</i> c8	2004	Furfural and HMF	F Coniochaetales	Lopez et al. (2004)
<i>Methylobacterium extorquens</i>	2004	Furfural and HMF ^b	– Rhizobiales	Lopez et al. (2004)
<i>Pseudomonas</i> sp.	2004	HMF ^a	– Pseudomonadales	Lopez et al. (2004)
<i>Acinetobacter</i> -like	2004	Furfural ^a	– Pseudomonadales	Lopez et al. (2004)
<i>Flavobacterium indologenes</i>	2004	Furfural and HMF ^b	– Flavobacteriales	Lopez et al. (2004)
<i>Stenotrophomonas maltophilia</i>	2004	Furfural and HMF ^b	– Xanthomonadales	Lopez et al. (2004)
<i>Desulfovibrio</i> sp.	1991	Furfural ^c	– Desulfovibrionales	Boopathy and Daniels (1991)
<i>Pseudomonas putida</i> Fu-1	1989	Furfural	– Pseudomonadales	Koenig and Andreesen (1989)
<i>Escherichia coli</i> K-12 NAR30, NAR40	1986	2-Furoic acid	– Enterobacteriales	Abdulrashid and Clark (1987)
<i>Desulfovibrio</i> sp. strain f-1	1983	Furfural ^c	– Desulfovibrionales	Brune et al. (1983)
<i>Pseudomonas putida</i> F2	1969	2-Furoic acid	– Pseudomonadales	Trudgill (1969)
<i>Pseudomonas</i> sp. 5863	1964	2-Furoic acid	– Pseudomonadales	Kakinuma and Yamatodani (1964)

^a– Gram negative, + Gram positive, F fungus^bBased solely on substrate depletion^cAnaerobic

These authors isolated “numerous microorganisms” capable of utilizing furanic compounds as a carbon source, several of which formed glutamic acid from 2-furoic acid. Since this early report, a few dozen other furan-degrading microorganisms have been identified, the majority of which very recently (Table 1).

Most furan-degrading microorganisms have been isolated using classical enrichment on HMF and/or furfural as a carbon source (Abdulrashid and Clark 1987; Boopathy et al. 1993; Koenig and Andreesen 1989; Lopez et al. 2004; Trudgill 1969; Wierckx et al. 2010; Yu and Stahl 2008). Trifonova

and coworkers (Trifonova et al. 2008a, b) identified several furfural degraders in an attempt to isolate microorganisms that reduced the phytotoxicity of torrefied grass fibers, using an elegant assay based on the germination of lettuce seeds. The overview presented in Table 1 shows that most furanic compound-degrading microorganisms characterized thus far are aerobic Gram-negative bacteria, belonging to a relatively small number of genera. This suggests that furan catabolism can only be found among specialized microorganisms. It should be noted, however, that the list is strongly biased by our recent results (Koopman et al. 2010b), where we

identified various new furan-degrading bacteria based on the presence of genes encoding homologues of the furanic catabolic enzymes of *Cupriavidus basilensis* HMF14.

Remarkably, only three fungal furanic aldehyde degraders have been reported to date, two *Coniochaeta ligniaria* strains (Trifonova et al. 2008a; Lopez et al. 2004) and *Amorphotheca resinae* ZN1 (Zhang et al. 2010). This underrepresentation of eukaryotes may be caused by the fact that fast-growing bacteria are more likely to emerge from short-term enrichment cultures. Alternative experimental setups such as the long-term enrichment used by Zhang et al. (2010) may lead to the identification of a greater number of furanic aldehyde-degrading fungi. Reports on anaerobic degradation of furfural are equally scarce: only two microorganisms, both *Desulfovibrio* strains (Brune et al. 1983; Boopathy and Daniels 1991), are known to convert furfural anaerobically, producing acetic acid. Similarly, until recently very few reports existed on microorganisms capable of degrading HMF. This is likely to be attributable to the focus on furfural degradation, in agreement with the abundance of this furanic compound in nature and with its widespread utilization in industry.

Toxic effects of furanic compounds

Aldehydes in general are highly reactive molecules, giving rise to the formation of reactive oxygen species (ROS; Feron et al. 1991; Zaldivar et al. 1999). Furanic aldehydes are no exception in this respect, causing ROS-associated damage to proteins, nucleic acids, and cell organelles (Almeida et al. 2009; Zaldivar et al. 1999; Allen et al. 2010). In furanic aldehyde-challenged *Saccharomyces cerevisiae*, expression of genes related to general stress adaptation and tolerance, central metabolism, transport, and degradation of damaged proteins were upregulated, indicating a system-wide stress response (Lin et al. 2009; Ma and Liu 2010). In addition to ROS-induced oxidative damage, furanic aldehydes exert several specific effects such as inhibition of enzymes in primary metabolism Modig et al. 2002). This typically leads to a heavily increased lag phase (Almeida et al. 2009; Mills et al. 2009) that may be overcome only by considerably increasing the inoculum density (Heer and Sauer 2008; Yu and Stahl 2008; Roberto et al. 1996). Furthermore, many microorganisms reduce or oxidize furanic aldehydes to their alcohol, respectively, carboxylic acid forms to ameliorate their toxic effect (Almeida et al. 2007, 2009; Wierckx et al. 2010). The toxic aldehyde can be eliminated in this way, but the cofactors required for these conversions (NAD/NADH) may be depleted, again increasing the lag phase (Lin et al. 2009; Liu 2006; Almeida et al. 2007).

Biodegradation of furanic compounds

As outlined above, reduction or oxidation of furanic aldehydes is quite commonly observed, and similar defense mechanisms against all sorts of aldehydes are found throughout nature. The oxidation or reduction of furanic aldehydes should not be confused, however, with degradation. Often, it is unclear to which extent furanic compounds are truly metabolized. In several reports, it is merely established that the furanic aldehydes have disappeared, without mention of the metabolic fate of the corresponding alcohols or carboxylic acids. This gives rise to much confusion in literature regarding the metabolic capacity of furanic compound “degrading” microorganisms. Therefore, all the different forms of the furanic compound (alcohol, aldehyde, and carboxylic acid) should be carefully monitored in order to establish whether the furanic aldehydes are actually metabolized or only transformed into a less toxic form (Wierckx et al. 2010).

The first biochemical route for aerobic furfural degradation was proposed by P.W. Trudgill in 1969, for the furfural-degrading bacterium *Pseudomonas putida* F2. This pathway was later verified and amended by Koenig and Andreesen (1990) and Koopman et al. (2010b). In the “Trudgill pathway” (Fig. 1b), furfural is first oxidized to 2-furoic acid by an aldehyde dehydrogenase (Koenig and Andreesen 1990). 2-Furoic acid is subsequently ligated to coenzyme-A by a furoyl-CoA synthetase, after which furoyl-CoA is hydroxylated at the C₅ position by a furoyl-CoA dehydrogenase. The resulting enol-CoA tautomerizes to its keto form, which has a lactone ring structure. The lactone ring opens through (spontaneous or catalyzed) hydrolysis, and after another keto-enol tautomerization, 2-oxoglutaroyl-CoA is formed. Through hydrolysis of the CoA thioester 2-oxoglutarate is released, which is metabolized via the tricarboxylic acid cycle.

In 1989, Koenig and Andreesen demonstrated the validity of parts of this pathway at the enzyme level in *P. putida* Ful (Koenig and Andreesen 1989). The furoyl-CoA dehydrogenase was shown to be dependent on molybdenum, by labeling with the molybdenum antagonist [¹⁸⁵W] tungstate while molybdate was present in the media. The formation of 2-oxoglutarate as the end product of this degradation pathway was confirmed by the addition of arsenite, which specifically inhibits 2-oxoglutarate dehydrogenase (Koenig and Andreesen 1990; Koopman et al. 2010b).

Recently, we have demonstrated that *C. basilensis* HMF14 utilizes the Trudgill pathway for 2-furoic acid metabolism. Moreover, we were able to fully characterize this pathway at the genetic level (see below; Koopman et al. 2010b). In addition to the enzyme activities characterized

(5-hydroxymethyl)-2-furoic acid degradation “modules” have been recruited later in evolutionary history, constituting complete degradation pathways for furfural and HMF by complementing existing common aldehyde detoxification routes.

Genetic background of furanic degradation routes

Identification of genes involved in furanic aldehyde degradation

Although the degradation pathway for furfural was partially characterized at the enzyme level in the late 1980s, the genetic background has only recently been elucidated. Nichols and Mertens (2008) provided some preliminary insights by analysis of *P. putida* Fv1 transposon mutants that were no longer able to grow on 2-furoic acid. Two mutants were obtained in which either *psfB* or *psfF* was disrupted. The *psfB* gene encodes a lysR-type transcriptional regulator and is surrounded by the *psf2* gene cluster. This cluster contains several genes, encoding a.o., an aldehyde dehydrogenase, and a xanthine dehydrogenase accessory factor that were proposed to have a role in furfural metabolism. However, a complete metabolic route could not be reconstructed. The *psfF* gene encodes a GcvR-family transcriptional regulator and is surrounded by the *psf9* gene cluster, which is involved in general stress tolerance (Nichols and Mertens 2008).

By a similar approach of transposon mutant screening and nucleotide sequence analysis, we identified the two gene clusters in *C. basilensis* HMF14 that are responsible for the degradation of 2-furoic acid and the conversion of HMF to 2-furoic acid (Koopman et al. 2010b). The “furfural cluster” contains the *hmfABCDE* genes, encoding all the enzyme activities proposed by Trudgill (1969) and Koenig and Andreesen (1990) as well as the newly identified CoA thioesterase. The *hmfFGH* gene cluster is involved in the metabolism of HMF to 2-furoic acid, via the key intermediate FDCA, as described in the previous paragraph. The ability to utilize HMF and furfural could be transferred to *P. putida* S12 by introducing the eight genes of the HMF and furfural clusters, confirming the functionality of the encoded enzymes. Furthermore, this unequivocally demonstrated that all essential genes for the metabolism of HMF and furfural had been identified (Koopman et al. 2010b).

Genetic organization of the genes involved in furanic compounds degradation

The availability of the genetic information included in the *hmf* clusters allowed the identification of various other

bacteria capable of degrading furfural and/or HMF (Fig. 2; Koopman et al. 2010b). When comparing the genetic organization of the *hmf* genes in these bacteria, the order of the *hmfABCDE* genes is highly conserved.

The sequence similarity levels of individual *hmfABCDE* homologues in confirmed furfural degraders suggested a rather relaxed sequence–functionality relationship. Among the furfural degraders, the lowest homology to HmfA, the major subunit of the putative furoyl-CoA dehydrogenase, was observed for *Dinoroseobacter shibae* DFL12 (54% identity). Apparently, even a moderate sequence similarity combined with the proximity of genes encoding a CoA synthetase, a molybdenum-dependent dehydrogenase and a CoA thioesterase can provide a good prediction of the furanic aldehyde-degrading capacities of strains previously not associated with this trait. Based on this observation, we extended the homology search of Koopman et al. (2010b) using somewhat less stringent search criteria. Figure 2 shows an updated and extended overview of the homology search, which now includes several Gram-positive bacteria. Among these, preliminary results indicate that *Geobacillus kaustophilus* HTA426 (Takami et al. 2004) can degrade furfural (unpublished data). In some of these Gram-positive putative furfural degraders, the structural order of the *hmfABCDE* homologues was different from the previously identified bacteria. Furthermore, more genes encoding enzymes with putative activities related to the furoyl-CoA dehydrogenase were found. It is known, however, that the domain organization of this type of molybdenum-dependent dehydrogenases can be quite variable (Kisker et al. 1998). Therefore, an alternative genetic organization does not necessarily imply a different catalytic activity, although the 2-furoic acid degradation capacities of the identified Gram positives remain to be confirmed.

The *hmfFGH* cluster is much less conserved than the *hmfABCDE* cluster, both with regard to its spatial organization and with regard to homology of the encoded amino acid sequences (Koopman et al. 2010b). Some *Burkholderia* and *Methylobacterium* strains have all *hmf* genes organized in a single large cluster. However, in most (putative) HMF degraders, the *hmfFG* and *hmfH* genes are organized in a diverse manner. The *hmfABCDE* genes encode the core 2-furoic acid pathway, which is also required for HMF degradation. Therefore, a high level of conservation may be expected for these genes, whereas the genes for HMF degradation may have been recruited later from other, possibly diverse, sources.

Additional genes related to furanic aldehyde degradation or tolerance

In addition to the genes encoding the enzymes that constitute the furfural and HMF metabolic pathways,

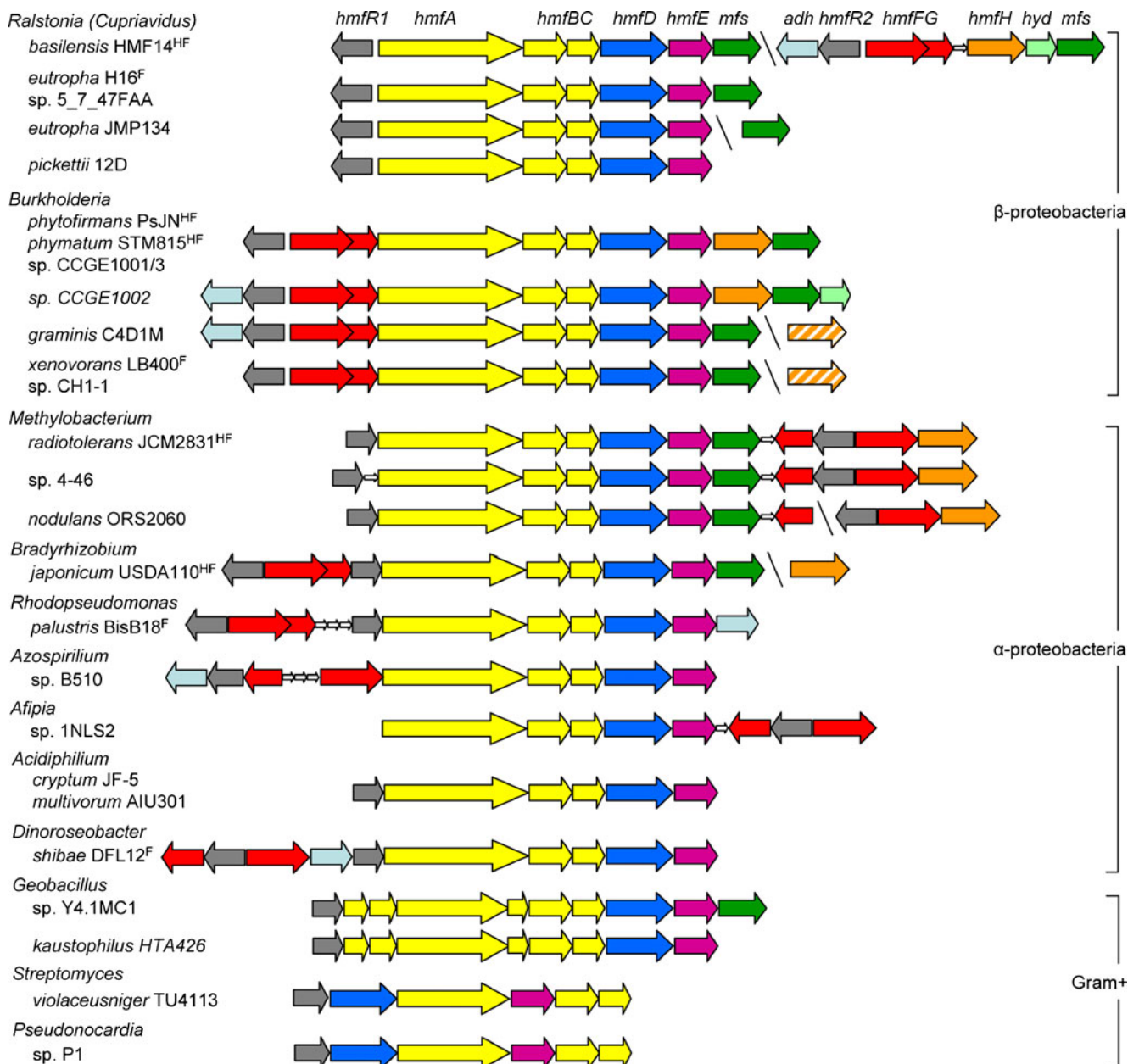


Fig. 2 Spatial organization of the furfural and HMF gene clusters of potential and established furfural and/or HMF degrading bacteria. Adapted from Koopman et al. (2010b). Species were included in the list based on the presence of genes encoding homologues of HmfA, HmfD and HmfE, with at least 40% similarity to the corresponding protein of *C. basilensis* HMF14, in each others' immediate vicinity. Gram-negative bacteria are subdivided into α- and β-proteobacteria. The following colors indicate putative encoded enzyme activities: gray transcriptional regulator, yellow furoyl-CoA dehydrogenase, blue

furoyl-CoA synthetase, purple oxoglutarate-CoA thioesterase, green major facilitator superfamily transporter, light blue aldehyde dehydrogenase, red 2,5-furandicarboxylic acid decarboxylase, light green hydroxylase, orange HMF/furfural oxidoreductase, white arrows indicate genes with no relation to furfural or HMF metabolism. Superscript letters indicate strains which have been confirmed to degrade furfural (^F) or both HMF and furfural (^{HF}). Dashed orange arrows indicate putative HMF/furfural oxidoreductases which group into cluster 2 of Fig. 3

several additional genes have been identified that may be associated with furfural degradation or tolerance. In close proximity to the *hmf* genes of *C. basilensis* HMF14 are the *mfs*, *hmfR*, *adh*, and *hyd* genes. Homologues of these genes can also be found in proximity to the *hmf* genes of the various species depicted in Fig. 2. These genes are

not essential for the degradation of HMF or furfural, but may play a role in transport, transcriptional regulation, and possibly redundant metabolic functions.

The latter may be true for the homologues of the *adh* gene of *C. basilensis* HMF14 (Fig. 2, light blue) that are found in the vicinity of the *hmf* genes in several species.

The putative aldehyde dehydrogenases encoded by these genes show 59–69% identity to the PsfA aldehyde dehydrogenase of *P. putida* Fu1 (Nichols and Mertens 2008). These genes may well contribute to the oxidation of furfural and/or HMF. Several genes have also been identified in *S. cerevisiae* and *Escherichia coli* that are responsible for the reduction of furanic aldehydes (Petersson et al. 2006; Gutiérrez et al. 2006).

In the mutant analyses of furanic compound-degrading microorganisms, several additional genes with similar functions were identified (Koopman et al. 2010b; Nichols and Mertens 2008). Examples are genes encoding peroxiredoxins and proteases that may be related to a general aldehyde-induced stress response (Zaldivar et al. 1999). Such genes may prove to be interesting targets for increasing the furanic aldehyde tolerance of industrial production hosts such as *S. cerevisiae* or *E. coli*.

Phylogeny of putative HMF/furfural oxidoreductase enzymes

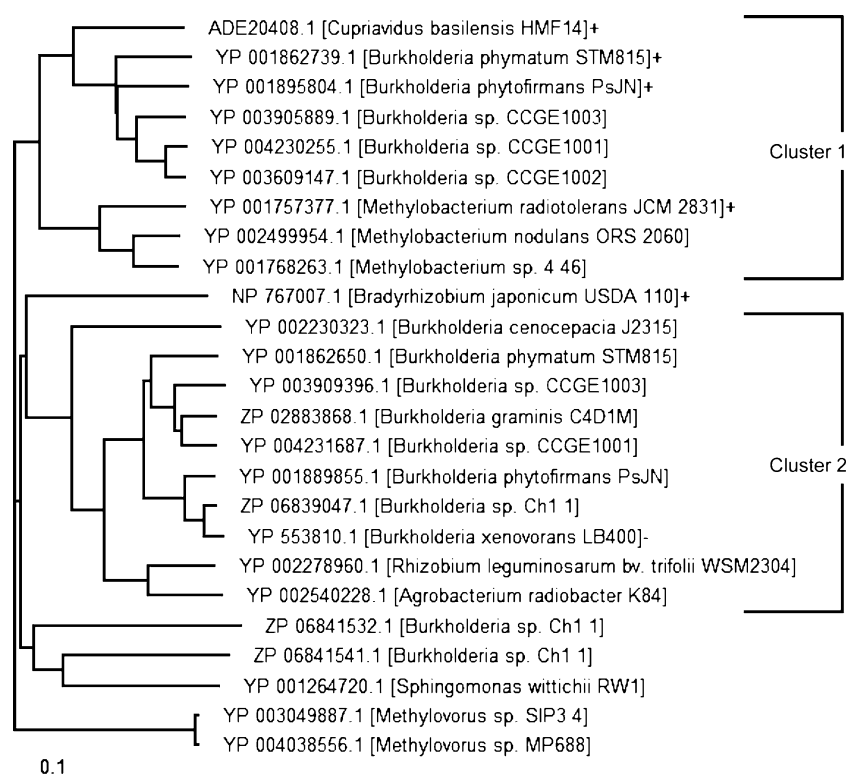
Although the HMF metabolic pathway consists of only two specific activities, the HmfH enzyme seems to be the determining factor for HMF degradation. Several strains which are incapable of HMF degradation possess homologues of *hmfFG*. Vice versa, strains that are incapable of HMF degradation never possess a functional homologue of *hmfH*. Therefore, the presence of an *hmfH* homologue

seems to be representative for the capacity to degrade HMF. *Burkholderia xenovorans* LB400 appears to be an exception, since it apparently contains both *hmfH* and *hmfFG*, but is unable to degrade HMF. Therefore, different HmfH homologues were analyzed in further detail by ClustalW2 cluster analysis. From this analysis, it is evident that two distinct clusters can be discriminated (Fig. 3).

Cluster 1 contains the HmfH homologues of established HMF-degrading bacteria, in addition to several bacteria whose HMF-degrading capacity has not yet been confirmed. The putative HmfH from *B. xenovorans* LB400, on the other hand, belongs to Cluster 2. Although this strain is the only confirmed non-HMF degrader, Cluster 2 also includes HmfH homologues of species that do not even possess significantly similar HmfABCDE homologues. As these are a prerequisite for HMF degradation, it appears likely that Cluster-2 HmfH homologues are not associated with the ability to degrade HMF. However, the correlation between the clustering behaviour of HmfH homologues and the (in-)ability to utilize HMF should be further confirmed.

It may be noted that some *Burkholderia* strains contain both a Cluster-1 and a Cluster-2 HmfH homologue. Presumably, the Cluster-1 HmfH is responsible for its ability to utilize HMF, since genes encoding this Cluster-1 HmfH are all in close proximity to the rest of the *hmf* genes (Koopman et al. 2010b). The HmfH homologue of *Bradyrhizobium japonicum* USDA 110 presents an exceptional case. This strain does degrade HMF, but its HmfH

Fig. 3 Cluster analysis of putative HMF/furfural oxidoreductases. The neighbor-joining plot was generated by TreeView (Page 1996) from a ClustalW2 alignment (Thompson et al. 2002) of all known proteins having at least 40% identity to HmfH from *C. basiliensis* HMF14. The scale bar represents 0.1 substitutions per nucleotide position. *Positive symbol behind the protein name* indicates that this protein has been implicated in HMF degradation. *Negative symbol* indicates a protein in a species able to degraded furfural, but not HMF (Koopman et al. 2010b)



homologue does not fall within Cluster 1. However, clustering with Cluster 2 is also poor, suggesting this enzyme may have to be considered an atypical example of an HMF oxidoreductase.

Conclusions: impact and future prospects

Biological detoxification

The study of the microbial metabolism of furanic aldehydes has led to a number of new possibilities, mostly related to the use of lignocellulosic hydrolysates. Most studies performed to date have focused on the removal of furanic inhibitors from lignocellulosic hydrolysate using microorganisms that metabolize furfural and/or HMF (Nichols et al. 2008, 2010; Okuda et al. 2008; Wierckx et al. 2010; Parawira and Tekere 2011). Although this process of bioabatement holds great promise for increasing the fermentability of lignocellulosic hydrolysate, several drawbacks exist related to the types of microorganisms used. Most bioabatement processes described to date are performed aerobically at near-neutral pH. Aerobic detoxification may be compatible with, or even be beneficial for, subsequent anaerobic fermentation. However, the high amount of energy required for aeration of hydrolysates (which often have a high solids content), and the risk of infection at near neutral pH, pose a serious threat to the economic feasibility at an industrial scale. Additionally, most bioabatement organisms described also consume the sugar fraction which is highly undesirable unless the bioabatement organism is also the production host, as described by Yu and Stahl (2008).

A truly efficient bioabatement process would require an organism that consumes only inhibitors such as furanic aldehydes, preferably at low pH and under anaerobic conditions. Since such organisms are not available, these must either be retrieved from nature or engineered by implementing a furanic aldehyde degradation pathway in an anaerobic acidophile. Obviously, such a pathway may also be implemented directly in an industrial production host to enable simultaneous detoxification and fermentation. This approach would perfectly match a consolidated bioprocess setup, uniting not only biomass hydrolysis and fermentation in a single microorganism, but also removal of inhibitors that emerged from biomass pretreatment.

Opportunities and limitations for heterologous expression of furanic aldehyde catabolic pathways

As described above, we have functionally expressed the furfural and HMF pathways of *C. basilensis* HMF14 in *P. putida* S12 (Koopman et al. 2010b). This demonstrated that

the furanic aldehyde pathways can—in principle—be applied in a heterologous host. By this approach, the efficiency of utilizing lignocellulosic hydrolysate as a biotechnological feedstock may be improved, since it would allow the microbial host to metabolize the toxic furanic aldehyde inhibitors.

It should be noted, however, that the specific properties of certain enzymes in the pathways pose limitations to their applicability. Based on homology to other enzymes, it is expected that the furoyl-CoA dehydrogenase encoded by *hmfABC* requires a specific molybdopterin cofactor (MoCo), the exact type of which is presently unclear. This limits the applicability of the furaldehyde catabolic pathways to hosts that possess the correct molybdopterin biosynthesis pathway. Although MoCos are widespread cofactors, the major industrial host *S. cerevisiae* lacks a molybdopterin biosynthesis pathway (Unkles et al. 1999). Implementation of the furanic aldehyde pathway in this host would therefore require the additional engineering of MoCo biosynthesis, which is a complex trait involving a large number of genes (Schwarz 2005). Furthermore, the oxygen dependency of the HMF/furfural oxidoreductase limits its application to aerobic processes, whereas most industrial fermentation processes are performed under micro-aerobic conditions at best.

Production of FDCA

As described above, oxygen dependency of the HMF/furfural oxidoreductase limits its applicability in anaerobic detoxification. However, this unique enzyme may find industrial application for the production of FDCA. FDCA has been proclaimed by the US Department of Energy as one of a list of 12 promising biobased chemicals that should contribute to the “greening” of the chemical industry in the near future (Werpy and Peterson 2004). Through expression of the *C. basilensis* oxidoreductase in solvent-tolerant *P. putida* S12, we were able to obtain 30 g/l of FDCA from HMF at a yield of 97% (Koopman et al. 2010a). FDCA is regarded as a biobased alternative to terephthalic acid for the production of polyethyleneterephthalate and other aromatics containing polymers, as well as an important platform compound for the synthesis of various chemical building blocks (Werpy and Peterson 2004; Bozell and Petersen 2010). As such, FDCA promises to be of great economic, as well as environmental benefit.

Future prospects

So far, the furfural and HMF degradation pathways of *C. basilensis* HMF14 constitute the only fully elucidated metabolic routes for these compounds (Koenig and Andreesen 1990; Koopman et al. 2010b; Trudgill 1969).

It can obviously not be excluded that furanic compounds can be degraded via alternative routes. The observation that mostly relatively similar bacteria were identified as furan degraders based on the genetic information of the *hmf* genes of *C. basilensis* HMF14, as opposed to other established furan degraders, supports the latter view. It is therefore expected that the characterization of the metabolism of furanic compounds in fungi or anaerobic bacteria will lead to the discovery of alternative metabolic pathways. These will certainly make useful additions to the existing toolbox of furaldehyde degradation pathways that may not only help to overcome the limitations of the *C. basilensis* HMF14 pathway for industrial application, but also provide new opportunities for the biocatalytic production of furanic compound-based fine chemicals and building blocks.

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Conflict of interest The authors declare that they have no conflict of interest.

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