Galactofuranose-Related Enzymes: Challenges and Hopes
Mateja Seničar, Pierre Lafite, Svetlana Eliseeva, Stephane Petoud, Ludovic Landemarre, Richard Daniellou

To cite this version:
Mateja Seničar, Pierre Lafite, Svetlana Eliseeva, Stephane Petoud, Ludovic Landemarre, et al.. Galactofuranose-Related Enzymes: Challenges and Hopes. International Journal of Molecular Sciences, MDPI, 2020, 21 (10), pp.3465. 10.3390/ijms21103465 . hal-02922109

HAL Id: hal-02922109
https://hal.archives-ouvertes.fr/hal-02922109
Submitted on 16 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Galactofuranose-Related Enzymes: Challenges and Hopes

Mateja Seničar 1,2, Pierre Lafite 1, Svetlana V. Eliseeva 2, Stéphane Petoud 2, Ludovic Landemarre 3 and Richard Daniello 1,*

1 Institut de Chimie Organique et Analytique, CNRS UMR 7311, Université d’Orléans, Rue de Chartres, BP 6759, CEDEX 2, 45067 Orléans, France; mateja.senicar@univ-orleans.fr (M.S.); pierre.lafite@univ-orleans.fr (P.L.)
2 Centre de Biophysique Moléculaire, CNRS UPR 4301, Rue Charles Sadron CS 8005, 45071 Orléans, France; svetlana.eliseeva@cnrs.fr (S.V.E.); stephane.petoud@cnrs-orleans.fr (S.P.)
3 GLYcoDiag, 45200 Chevilly, France; landemarre@glycodiag.com
* Correspondence: richard.daniello@univ-orleans.fr; Tel.: +33-238-494-978

Received: 19 April 2020; Accepted: 11 May 2020; Published: 14 May 2020

Abstract: Galactofuranose is a rare form of the well-known galactose sugar, and its occurrence in numerous pathogenic micro-organisms makes the enzymes responsible for its biosynthesis interesting targets. Herein, we review the role of these carbohydrate-related proteins with a special emphasis on the galactofuranosidas we recently characterized as an efficient recombinant biocatalyst.

Keywords: glycobiology; galactofuranosyl transferase; galactofuranoside hydrolase; pathogen diagnostics; carbohydrate-based therapeutics

1. Introduction

While the existence of less common l-galactose was only reported in some plants, algae and snail glycans, d-Galactose is a hexoaldose, C-4 epimer of glucose, widely distributed in nature and it can be found in both pyranose and furanose configurations [1]. Indeed, in both solid and solution states, hexoses exist in a cyclic hemiacetal form and occupy either the most sterically and thermodynamically favored six-membered pyranosyl or five-membered furanosyl forms. Both cyclic forms of galactose, Galβ and Galp, are interconverted through a linear form, which is present only in an instantaneous extent (Figure 1) [2].

Figure 1. Representation of the equilibria between cyclic forms of d-galactose in solution. The rapid interconversion of cyclic Galβ and Galp takes place through an acyclic form, and the favorable equilibrium for Galp is indicated by the arrows [3].

Nevertheless, galactofuranose is by far the most widespread hexofuranose in nature and its occurrence was recently reviewed [4]. This thermodynamically disfavored hexofuranose is absent in
mammalian glycoconjugates, but is present in living organisms ranging from archaea and bacteria, to protozoa, fungi and plants where it forms glycosidic linkages mainly in β-anomeric configuration, although rare examples in which an α-anomeric configuration occurrence can also be found [5]. In mammals, only the galactopyranose is found as a ubiquitous form. Exceptionally, it can also be present as free monosaccharide in nature where it is commonly linked to other molecules like glucose, constituting simple disaccharide structures (lactose or milk sugar), or as a constituent of glycans in complex glycoproteins (ABO blood group antigens) and glycolipids [1,6].

The interest for furanoses dates back to the beginning of the 20th century, and the beginning of the carbohydrate chemistry occurred when the first synthetic methods for the selective synthesis of glycofuranosides were established [7]. Galactofuranose was first identified in 1937 as a component of a fungal extracellular polysaccharide, galactocarolose, produced by Penicillium charlesii [8,9]. Only years later, the synthesis of the first β-d-galactofuranosides was reported [10]. Over the decades, galactofuranose has been found in many naturally occurring molecules originating from a variety of organisms, not necessarily pathogenic. Lately, the interest for this unusual carbohydrate has not decreased due to findings of its xenobiotic and immunogenic properties [4]. In addition, its occurrence in pathogenic organisms make the enzymes related to its biosynthesis of outmost interest for the glycoscientists at large, as these proteins, if cloned, overexpressed and well-characterized, can be further used as innovative biocatalysts, therapeutic targets or for diagnosis. All these aspects will be discussed herein.

2. Occurrence in Nature

In the following sections, the occurrences of β-d-Galβ units will be presented. Here, the intention is not to be exhaustive and to provide a complete overview of all natural Galβ-containing structures, but rather to focus on major structures, particularly found in prokaryotes and in two different classes of eukaryotes, fungi and protozoa and to illustrate them with the representative and most studied examples. Thereby, the focus will mainly be on pathogenic organisms such as bacteria Mycobacterium tuberculosis, fungus Aspergillus fumigatus and protozoa Leishmania major.

2.1. In Bacteria

In both Gram-positive and Gram-negative bacteria, Galβ is either part of a homopolymer formed with a Galβ disaccharide as the repeating unit, or part of a heteropolymer, linked to another monosaccharide, frequently galactopyranose, forming regular glycans that are sometimes branched [5]. These glycans, composed of Galβ units, are usually part of complex glycoconjugates structures that are constituents of bacterial cell walls. Some of these bacteria are highly pathogenic, and the presence of Galβ-containing conjugates appears to be an essential parameter related to their virulence. These conjugate include lipopolysaccharide (LPS) O-antigens of Escherichia coli K-12 and enteroinvasive E. coli O164 as well as the galactan-I repeating unit of O-antigen from Klebsiella pneumoniae [4,6].

The notable example of highly complex structure constituted largely of rare carbohydrates and lipids is the mycobacterial cell envelope where Galβ has a crucial constructive role [11]. After several decades of successful chemotherapeutic treatments and vaccination preventions, Mycobacterium tuberculosis has emerged as being multidrug resistant, and tuberculosis became one of the major causes of mortality worldwide [4,12]. The cell envelope of Mycobacterium tuberculosis is a very thick, hydrophobic structure possessing very limited permeability. It consists of three main structural components: typical prokaryotic plasma membrane, cell wall and outer membrane, also referred as mycomembrane [13]. The entire complex of cell wall is noncovalently attached to plasma membrane with the bottom peptidoglycan layer, which, via a disaccharide linker, connects to the central glycan core, highly branched arabinogalactan polysaccharide. In addition, two-thirds of the arabinans are terminated by a cluster of mycolic acids that form outer membrane layer and capsular segment containing a variety of loosely attached proteins, lipids and polysaccharides (Figure 2) [6,13].
Arthrobacter sp., contains a repeating trisaccharidic motif and shows antitumor activity [4,18].

Lactobacillus rhamnosus positioned unit in the glycan core motifs [5]. It is the case in the anaerobic eubacteria Galactomannan is a main exopolysaccharide structural form of the cell wall, composed of (1\(\rightarrow\)3)-D-Manp and (1\(\rightarrow\)6)-D-Galf [3].

In numerous pathogenic fungi species, for example: Aspergillus, Histoplasma, Cryptococcus and Aspergillus fumigatus causes aspergillosis, one of the main fungal infections in humans, especially in immunodeficient patients. Interestingly, a single Galf is essential for the immunoreactivity of the glycan and therefore they have been studied as in vitro markers for the early diagnosis of the invasive aspergillosis [5]. It is remarkable since 5% of the dry weight of A. fumigatus consists of Galf, and as it is a quite abundant monosaccharide found and described at least in four different molecules, i.e., galactomannan and its secreted form, glycoproteins, and within glycosphingolipids [3]. Galactomannan is a main exopolysaccharide structural form of the cell wall, composed of (1\(\rightarrow\)2)- and (1\(\rightarrow\)3)-D-Manp.
(1→6)-α-d-mannopyranoside core chain with around four to ten units of (1→5)-β-d-Galf residues. This galactomannan is also secreted and can be found as a free, soluble polysaccharide in the medium. In addition, β-Galf-(1→5)-β-Galf was identified in both O- and N-linked glycans from the peptidogalactomannan and glycoinositolphosphoceramide [21,22].

![Diagram of cell wall structure](image)

Figure 3. A schematic representation of the *Aspergillus fumigatus* cell wall structure with a depiction of the three major cell wall features, including chitin, glucan and galactomannan [14,23].

Other, noteworthy examples of d-Galf-containing molecules present in other fungal species, include the less common α-d-Galf found together with β-d-Galf in varianose, extracellular polysaccharide produced by *Penicillium varians* [24]. The same repeating units of varianose have been found in the cell wall of exopolysaccharides of *P. vermiculatum* and *Talaromyces flavus* while *Apodus deciduus* contains the (1→2)-Galf disaccharide only in α-configuration [5].

2.3. In Protozoa

The presence of Galf in eukaryotic protozoa was reported in the early 1980s in the family of *Trypanosomatidae*, which includes for humans and animals parasitic genera *Trypanosoma* and *Leishmania*. The glycoconjugates in these parasites have been characterized in detail and to our knowledge, no other examples of protozoa except trypanosomatids *Crithidia* spp. and *Endotrypanum schaudiinii* containing Galf have been reported [25,26].

Protozoan parasites of the genus *Leishmania* have two different life cycle stages, the flagellate promastigote stage in insects and the amastigote stage in mammalian phagocytic cells, during which many morphological and structural changes of the glycoclayx membrane occur. The related Galf glycosylated membrane macromolecules are well studied in *Leishmania major*, a causative agent of leishmaniosis, and include two types of molecules, lipophosphoglycans (LPGs) and glycoinositolphospholipids (GIPLs). LPG is a major glycoconjugate that covers entire surface of promastigote and contains an internal hexasaccharide β-Galf(1→3)-Manp core, which is conserved among all *Leishmania* species. The GIPLs are present at the membrane surface at a ten times higher amount than LPGs, and contain the same repeating unit, only externally positioned. Both glycolipid complexes, LPG and GIPL, are involved in the virulence and survival of the parasite, and their unconventional hexasaccharide core has become a target for the search for new drugs [27,28]. Similar LPG and GIPL are present in *Trypanosoma cruzi*, the causative agent of the Chagas disease [4].

2.4. In Other Organisms

Beside previously mentioned groups of organisms, other eukaryotic organisms expressing different Galf-containing surface molecules include certain plants, lichens and marine organisms. Even if rare in terrestrial plants, Galf was first described in 1981 as a part of the cell wall glycoproteins in the...
unicellular green algae Chlamydomonas reinhardii [29]. Molecularly unique, prymnesin-1 toxin, isolated from red tide alga Prymnesiou parvum, contains β-D-Gal residue linked to C₉₀ unbranched carbon chain (Figure 4A) [4,30].

Figure 4. Chemical structures of Galf-containing glycoconjugates. (A) The structure of toxin prymnesin-1, produced by Prymnesiou parvum. (B) The structure of agelagalastatin, glycosphingolipid produced by Agelas sp. [4].

Among marine organisms, especially sponges of the genus Agelas sp. glycosphingolipid agelagalastatin was described as containing the trisaccharide α-Galf(1→2)-β-Galf-(1→3)-Galp, and it showed an antitumoral activity (Figure 4B) [31]. Steroidal glycosides and gangliosides containing Galf were isolated from the starfish Anthenea chinensis and Achanthaster planci respectively [4,5]. The presence of Galf was also reported in the nematode Caenorhabditis elegans, one of the model organisms in molecular and animal biology studies, but no reports about the presence in the mammals, including humans, have been reported so far [32].

3. Aspects of Enzymatic Biosynthesis and Metabolism

The uniqueness of Galf as a central component of cell surface glycoconjugates of human pathogens has led to an increased interest for the elaboration of its biosynthesis. The detailed mechanism of its biosynthesis and metabolism has been difficult to elucidate due to the instability of Galf itself
and to the fact that only few of these enzymes have been isolated and studied in the purified form. From the studies available so far, it is evident that two putative enzymes, UDP-galactopyranose mutase (UGM) and galactofuranosyltransferase (GalfT) catalyze two synthetic steps and give a rise to a galactofuran extracellular conjugates, while the exact function of galactofuranosidase (Galf-ase), as the degrading enzyme, and its overall contribution to the Galf metabolism remains unclear (Figure 5) [3,6]. In addition to the confirmation of the canonical metabolism scheme and of the absence of alternative pathways, the identification of the three classes of enzymes involved and the coding genes, the efforts to understand their in vivo interaction and importance for viability or virulence, possibly in one organism, are still ongoing.

![Figure 5. Schematic representation of the Galf-glycan biosynthesis and the involvement of the three main enzymes, UDP-galactopyranose mutase (UGM), galactofuranosyltransferase (GalfT) and galactofuranosidase (Galf-ase).](image)

**3.1. UDP-Galactopyranose Mutase (UGM)**

Generally, furanoses, as nucleotide activated monomer donors, are transferred to the acceptors, growing oligomers or polymers, by glycofuranosyltransferases. In vivo, these furanose donors are generated from the corresponding pyranoses by the activity of pyranose mutases. The sole source of all Galf residues is uracil diphosphate (UDP)-galactofuranose (UDP-Galp), which originates directly from the UDP-galactopyranose (UDP-Galp). In addition, UDP-Galp is biosynthesized in all species from glucose, originating from a de novo synthesis pathway, or in some organisms it may be formed from free galactose by galactose salvage pathway. The activated UDP-Galp is interconverted, through reversible ring-contraction, into UDP-Galf by the cytosolic enzyme UDP-Galp mutase. The equilibrium of the UGM-catalyzed reaction greatly favors thermodynamically more stable UDP-Galp by the 11:1 ratio (Figure 6) [1,33].

![Figure 6. UGM-catalyzed isomerization of UDP-Galp and UDP-Galf.](image)

It was established first in 1971 in *Salmonella typhimurium* that UDP-Galp is precursor of UDP-Galf. More than twenty years later, in 1996, the *g*lf gene encoding for a UGM was first identified and cloned from *E. coli* [34]. Soon after the existence of homologue genes and UGMs was confirmed and characterized in several other procaryotes, bacteria *Klebsiella pneumoniae*, *Mycobacterium tuberculosis* and *Campylobacter jejuni*, as well as in protozoan eukaryotes *Leishmania major* and *Trypanosoma cruzi*, fungus *Aspergillus fumigatus* and nematode *Caenorhabditis elegans* [1,35].

In 2001, the first crystal structure of UGM from *E. coli* was reported [35]. Currently, a total of 58 crystal structures of UGM obtained from nine organisms have been deposited in the Protein Data Bank (PDB). Some of these structures have been crystalized in both, the active (reduced) and the
inactive (oxidized) states, as well as complexed with different substrates or ligands. The structures
gave insight into its tertiary structure and revealed that the overall architecture of active site consists of
conserved amino acid residues and that generally, UGMs are flavoenzymes working by the unique
mechanism involving flavine adenine dinucleotide (FAD) cofactor in its reduced form [35,36].

Since the UGM is at the center of Galf biosynthesis, the mutagenesis or the deletion of its genes
is enabling to study the impact of UGM absence and, consequently the Galf absence, on the in vivo
organism integrity.

3.2. Galactofuranosyltransferase (GalfT)

After the isomerization catalyzed by UGM, the newly produced UDP-Galf is transported, via
the UDP-Galf transporter only in eukaryotes, from the cytosol into the Golgi apparatus where the
glicosylation by galactofuranosyltransferases takes place. GalfTs are the final enzymes involved in the
biosynthesis of Galf-containing molecules by a catalyzed reaction that corresponds to the nucleophilic
substitution of an acceptor on the anomeric position of an activated sugar donor (Figure 7) [6].

Probably due to the high costs and difficulties in obtaining the activated donor combined with the
limited access of the relevant enzymes, GalfTs were less studied than the mutases [5]. However, at the
time, only a few GalfTs of prokaryotic, exclusively bacterial, and eukaryotic origin have been
cloned and characterized [1,5,6].

By far the most studied prokaryotic GalfTs are GlfT1 and GlfT2, two essential transferases for the
biosynthesis of Mycobacterium tuberculosis galactan. Interestingly, they share a low sequence homology
and are coded by different genes; GlfT1 is encoded by the Rv3782 gene and GlfT2 by the Rv3808c
gene, respectively. Both transferases have been expressed as recombinant enzymes and characterized.
The subsequent studies elucidated role of each transferase and demonstrated that GlfT1 is responsible
for the transfer of the two first Galf on the acceptor substrate, whereas GlfT2 pursues the polymerization
of the resulting acceptor by introducing approximately 30 remaining monosaccharides [37–39].

Since both use UDP-α-D-galactofuranose as the donor and the change from α-stereocchemistry in
UDP-Galf to the β-stereocchemistry of the newly synthesized glycan, indicate that catalysis follows an
inverting mechanism. They are also characterized as being bifunctional because they are synthesizing
both β-Galf-(1→5)-Galf and β-Galf-(1→6)-Galf linkages between Galf residues. GlfT2 has been more
extensively studied than GlfT1; therefore, it is the only GalfT with reported crystal structure and the
confirmed presence of only one catalytic site [38,39].

Few other well-characterized prokaryotic GalfTs include WbbI from Escherichia coli K-12 that is
able to transfer β-(1→6)-Galf to α-glucose [40] and WbbO from Klebsiella pneumoniae, another
bifunctional transferase that couples β-Galf-(1→6)-Galf [41].

Until recently, LPG1 from Leishmania major was the first and only described eukaryotic GalfT.
In an extensive study from 2018, all four putative transferases (LPG1, LPG1G, LPG1L and LPG1R)
encoded in the L. major genome were cloned, overexpressed and their kinetic parameters determined.
It was demonstrated that they are able to use both UDP-Galf and UDP-Galp as donor substrates [42].

Another two-known eukaryotic GalfTs are of fungal origin, GfsA from Aspergillus nidulans and
AfgsA from Aspergillus fumigatus. As it was shown, both enzymes are localized in the Golgi apparatus and use
UDP-Galf as a sugar donor [43,44]. Moreover the galactofuranosyltransferase activity was demonstrated
in *P. fellatum* through the incorporation of radiolabelled Gal\(^f\) into peptidophosphogalactomannan prepared from its membrane, but no further investigations were conducted [45].

### 3.3. Galactofuranosidases

Although the metabolism of β-\(\alpha\)-galactofuranosides has been extensively studied, mostly in the infectious microorganisms such as *Mycobacteria, Trypanosoma, Leishmania* and *Aspergillus*, there are only few reports related to these enzymes, especially galactofuranosidase Gal\(f\)-ase.

Over the past forty years, Gal\(f\)-ase has been identified as being responsible for the degradation of the \(d\)-Gal\(f\) containing glycoconjugates [5,6]. In 1977, a specific extracellular exo-β-\(\alpha\)-galactofuranosidase (exo-β-\(\alpha\)-Gal\(f\)-ase) was the first one isolated and partially purified from *Penicillium fellutanum* (ex type of *Penicillium charlesii*) culture filtrates. The enzyme catalyzed the hydrolysis of β-galactofuranosides only and was incapable of cleaving β-L-arabinofuranosides, the pentosyl homologs. The enzyme was stable to freezing and thawing and reached optimum activity between pH 3 and 4 at the temperature of 47 °C [46].

Later, extracellular exo-β-\(\alpha\)-Gal\(f\)-ases were described and/or purified from the culture medium of filamentous fungi such as *Helminthosporium sacchari* [47,48], *Trichoderma harzianum* [49], *Penicillium* and *Aspergillus* species [50], among which the one from *P. fellutanum* have been the most studied [46,51–53], *Aspergillus niger* [54] and protozoa *Trypanosoma cruzi* [55]. All the known β-Gal\(f\)-ases were exclusively exo enzymes and mostly have been of fungal origin.

There are only two reported endo-β-\(\alpha\)-Gal\(f\)-ases, isolated from fungus *Penicillium oxalicum* [56] and from bacteria *Bacillus* sp. [57]. The first endo-β-\(\alpha\)-Gal\(f\)-ase, purified in 1992 from supernatants of *P. oxalicum* autolysed cultures, hydrolyzed specifically β-\(\alpha\)-\(1\rightarrow5\)-linked galactofuranose residues. This was the first endo-β-\(\alpha\)-Gal\(f\)-ase for which optimum pH, stability properties, substrate specificity and kinetic characteristics were determined [56].

Only a few specific β-\(\alpha\)-Gal\(f\)-ases have been reported and mostly detected in fungal species as an extracellular enzyme, which is secreted into the medium in low quantities.

In the absence of the identified Gal\(f\)-ase gene and overexpressed as a recombinant protein, their production was challenging. In order to induce a higher level of enzyme secretion, the synthetic growth medium was supplemented with various monosaccharides, disaccharides or polysaccharides [51], sometimes with those containing galactofuranoside residues [54,57] or the media from natural sources, like apple juice [50] was used. The production of Gal\(f\)-ase in *A. niger* were induced to significant levels in the presence of Gal\(f\) containing glycoconjugates (mycelial wall extracts) [54] and in *P. fellutanum* and *A. fumigatus* when the medium was depleted of glucose [46,50,51,58] or when the glucose was replaced by galactose as the carbon source [59].

Mostly, Gal\(f\)-ases were purified from *P. fellutanum* culture filtrates and have become model enzymes in characterization studies [46,47], but Gal\(f\)-ase were also commercially available from crude enzyme preparations [49,60] or from *T. cruzi* cell lysates [55].

To detect low levels of Gal\(f\)-ase in culture media, to isolate it and to possibly purify it, an array of Gal\(f\)-containing glycoconjugates, acting like potential substrates or inhibitors, was synthetized and assayed. Several conjugates proved to be good inhibitors, such as alkyl, aryl and heteroaryl 1-thio-β-\(\alpha\)-galactofuranosides [59,61]. An affinity chromatography system was also developed using two inhibitors, 4-aminophenyl thio-β-\(\alpha\)-galactofuranoside and β-galactono-1,4-lactone, as the immobilized and eluting ligands [52].

These early studies presented the pioneering work in the Gal\(f\)-ase research. In most cases, properties were determined using partially purified or even crude enzyme preparations and one of the main problems was the lack of a simple and sensitive quantitative method for the detection of their catalytic activity as well as a standardized substrate. The activity was usually determined by measuring the released galactose during the hydrolysis of galactofuranose-containing exopolysaccharide preparations of natural origin or methyl β-\(\alpha\)-Gal\(f\) by the galactose oxidase method. Already experimentally established, the use of nitrophenyl glycosides, widely used as substrates for estimating the activity,
kinetics and specificity of glycosidases, was extended to the para-nitrophenyl \( \beta \)-d-galactofuranose (\( pNP-\beta\)-d-Galf) [50,62], which became a standardized and commercially available [54] substrate for assaying galactofuranosidases.

A colorimetric assay with \( pNP-\beta\)-d-Galf as a substrate was extensively used. However, this substrate was not recognized by the endo-\( \beta\)-d-Galf-ase from Bacillus sp. [57] and exo-\( \beta\)-d-Galf-ase from T. cruzi [55] and could be attributed to a particular substrate specificity, relating to aglycone.

The diversity of organisms, experimental conditions and substrates were employed during these research studies making it very difficult to establish a unanimous and general conclusion. However, they showed reliable evidence that suggested some common characteristics, which are outlined (Table 1). The substrate specificity in respect to the glycon moiety from either natural or artificial substrates was exclusively towards \( \beta\)-d-Galf. The enzymes proved to be stable over a longer period of time, even as crude preparations, and showed optimal activity in acidic conditions that are usual for the enzymes of invertebrates, (pH 4–5), and the stability at the temperatures up to 40 °C.

Although the first Galf-ase was described back in 1977, and several exo- and endo-Galf-ases were purified from the culture supernatants and cell lysates of filamentous fungi, bacteria and protozoa, the genes encoding these enzymes were not identified and expressed earlier, nor their amino acid sequence determined (Figure 8).

Recently, in 2015, based on the draft genome sequence analysis of soil, Gram-positive bacteria Streptomyces sp., strain JHA19, an open reading frame that encoded Galf specific enzyme was identified. Based on the sequencing results, the genome size was 7.7 Mb and the entire Galf-ase open reading frame (ORF) fragment contained 2361 base pairs (bp), which encoded 786 amino acids [63].

The Galf-ase gene fragment was cloned, expressed and purified as a recombinant Nus and double 6xHis-tagged fusion protein. The enzyme was described as an exo-type Galf-ase that hydrolyzed galactomannan, the naturally occurring Galf-containing oligosaccharide, extracted from A. fumigatus cell wall, as well as the artificial substrate \( pNP-\beta\)-d-Galf. No activity was observed with other \( pNP\) furanosyl and pyranosyl glycoconjugates, including \( pNP-\alpha\)-L-Araf. The optimal pH was found to be 5.5, and the enzyme was stable at temperatures up to 40 °C with the \( K_M \) value of 4.4 mM (Table 2).

This was the first report of an identification and cloning of a gene coding for the Galf-specific Galf-ase enzyme that does not also exhibit arabinofuranosidase (Araf-ase) activity [64].

---

**Figure 8.** Schematic representation of the timeline of Galf-ase discoveries and studies over the past forty years.
Table 1. Comparative properties of β-ν-galactofuranosidases.

| Enzyme                  | Species                        | Substrate                        | pH  | T (°C) | M (kDa) | K<sub>M</sub> (mM) † | Year | Reference |
|-------------------------|--------------------------------|----------------------------------|-----|--------|---------|-----------------------|------|-----------|
| extracellular           | Penicillium charlesii (fungus) | pPGM<sup>a</sup>                 | 4   | 47     | –       | –                     | 1977 | [46]     |
| exo-β-ν-GalF-ase        |                                |                                  |     |        |         |                       |      |           |
| β-ν-GalF-ase            | Helminthosporium sacchari (fungus) | 1-O-methyl-β-Galf                | 4.2 & 5.2 | 38     | –       | –                     | 1983 | [47]     |
| β-ν-GalF-ase            | H. sacchari (fungus)           | HS toxin<sup>b</sup>             | 4.6 | 37     | –       | –                     | 1983 | [48]     |
| extracellular           | Penicillium spp.               |                                  |     |        |         |                       |      |           |
| β-ν-GalF-ase            | Aspergillus spp. (fungi)       |                                  |     |        |         |                       |      |           |
| endo-β-GalF-ase         | Penicillium oxalicum (fungus)  | β-(1→5)-galactofuran             | 5   | 37     | 77      | –                     | 1992 | [56]     |
| exo-β-ν-GalF-ase        | Trichoderma harzianum (fungus) | EPS<sup>c</sup>                  | 4–4.5 | 35–40  | 35      | –                     | 1992 | [49]     |
| endo-β-GalF-ase         | Bacillus sp. (bacteria)        |                                  | 6   | 37     | 67      | –                     | 1995 | [57]     |
| exo-β-ν-GalF-ase        | Penicillium fellutanum (fungus) | pNP-β-ν-Galf<sup>f</sup>         | 3–6 | 37     | 70      | 0.3                   | 1999 | [52]     |
| extracellular β-ν-GalF-ase | Aspergillus niger (fungus)     | pNP-β-ν-Gal<sup>f</sup>          | 3–4 | 37     | 90      | 4                     | 2001 | [54]     |
| exo-β-ν-GalF-ase        | P. fellutanum (fungus)         | 1-O-methyl-β-Galf                | 4–4.5 | 40     | 70      | 2.6                   | 2001 | [53]     |
| exo-β-ν-GalF-ase        | Trypanosoma cruzi (protozoa)   | LPPG<sup>d</sup>                 | –   | –      | 55      | –                     | 2003 | [55]     |

† K<sub>M</sub> values determined only for pNP-β-ν-Galf and its derivatives as a substrate.  
a pPGM—peptidophosphagalactomannan from Penicillium fellutanum  
b HS toxin—host-selective toxin from Helminthosporium sacchari  
c EPS—extracellular polysaccharides from Penicillium digitatum  
d LPPG—lipopeptidophosphoglycan from Trypanosoma cruzi.
Another Galf-ase gene was also found later in the genome of *Streptomyces* sp., strain JHA26, coding for 869 amino acids. The Galf-ase specific enzyme was expressed, purified and characterized with the highest activity at pH 4.5 and temperature stability up to 45 °C and with $K_M$ of 6.8 mM for pNP-$\beta$-d-Galf as a substrate [65,66].

### Table 2. Comparative properties of recombinant $\beta$-d-galactofuranosidases.

| Galf-ase          | Substrate   | pH | T (°C) | $K_M$ (mM) | Year | Reference |
|-------------------|-------------|----|--------|------------|------|-----------|
| *Streptomyces* sp. (JHA19) | pNP-$\beta$-d-Galf | 5.5 | 50     | 4.4        | 2015 | [64]      |
| *Streptomyces* sp. (JHA26) | pNP-$\beta$-d-Galf | 4.5 | 45     | 6.8        | 2017 | [65]      |
| Galf-ase (JHA19)   | pNP-$\beta$-d-Galf | 4.5 | 60     | 0.25       | 2019 | [67]      |

The most recent addition of cloned enzymes is the one from 2019, which provided the complete biochemical and kinetic characterization of subcloned *Streptomyces* spp. JHA16 recombinant Galf-ase to date. This N-terminal 6xHis-tagged Galf-ase proved to be an efficient and stable biocatalyst exclusively towards the synthetic substrate pNP-$\beta$-d-Galf possessing a $K_M$ value of 0.25 mM and the highest activity at pH 4.5, temperature stability up to 60 °C as well as stability towards multiple freeze and thaw cycles as a crude preparation (Table 2) [67].

### 4. Galactofuranose Antigens—Therapeutic and Diagnostic Target

The search for molecules that are specific to pathogenic microorganisms, pathogen-associated molecular patterns (PAMPs), preferably surface-exposed, conserved in pathogens and absent in host organisms, to circumvent a potential risk of interference, has seemingly led to one potential target, the galactofuranose [68,69].

Biomolecules involving galactofuranose have attracted interest because they fulfill these requirements and their presence in many pathogens suggests to be an advantageous element of survival and is considered essential for their virulence [4]. Hence, galactofuranose is as a new and interesting candidate as a target in medical or biotechnological applications.

Possibilities to exploit the absence of this unusual monosaccharide in mammals arise from its biological significance in these pathogens. The impact of the galactofuranose deficiency on cell morphology and growth and its role in virulence was the focus of numerous research studies, predominately performed on eukaryotic pathogens of *Aspergillus* and *Leishmania* species. The modifications of the cell surfaces were most evident in fungi and resulted in aberrant morphological changes and growth reduction, leading to a hypersensitivity to drugs and osmotic stress. The lack of Galf had a variable impact on the virulence capacity of *Leishmania*, *Trypanosoma* and *Aspergillus* species. While in *Leishmania major*, Galf-deficient mutants presented only the initial delay in infection onset, in *L. mexicana* infectivity was not attenuated and in the related parasite *T. cruzi*, Galf-containing strains were less infectious than those expressing Galp. On the other hand *A. fumigatus* displayed attenuated virulence, which might be dose-related [3].

Interestingly, the virulence effects vary with the observed organisms, indicating that the Galf involvement cannot be generalized and rather should be considered separately, related to specific pathogen species. Understanding the exact role and contribution of individual Galf-containing glycoconjugates and Galf itself on the morphology, survival and virulence, as well as its role in the immune response, remain to be clarified.

Therefore, the medical application of Galf-based therapeutics or mimetics is still challenging and relies on the elucidation of the Galf biosynthesis pathways.

However, the importance of Galf as a diagnostic target showed it to be very useful. The presence of *Aspergillus* exoantigens of galactomannan (GM) origin, known to be secreted by the fungus during its growth in vitro and in vivo, is a specific indicator of this invasive disease and has become a detection target [70]. Monoclonal antibody detection methods for early serological diagnosis of galactomannan antigens, thus invasive pulmonary aspergillosis, have been experimentally developed since 1980s. It was not only until 1995 that a double-direct sandwich enzyme-linked immunosorbent assay (ELISA)
was developed, that this assay employs a rat anti-GM monoclonal antibody, EB-A2, directed against the \( \beta\text{-Gal}(1\rightarrow3)\beta\text{-Gal} \) epitopes of GM. Today, two GM antigen detection kits are commercially available, the Pastorex Aspergillus and the Platelia™ Aspergillus. The Pastorex, latex agglutination test, has mostly been replaced by the Platelia™ Aspergillus, EIA, which has been available in Europe for more than 20 years and in the USA since 2003. To our knowledge, these are the only commercially available tests based on the detection of Gal\( f \) epitopes [71,72].

In parallel, a novel experimental procedure for the non-invasive detection of Aspergillus lung infection, based on antibody-guided positron emission tomography and magnetic resonance (immunoPET/MR) imaging, has been developed and tested. In 2016 the prototype version, \([^{64}\text{Cu}]\text{DOTA-mJF5}\) tracer, showed that a mouse monoclonal antibody (mJF5) specifically binds to the mannoprotein antigen, pathogen related only, and that the antibody-labeled, radionucleotide \( ^{64}\text{Cu} \) and DOTA chelator complex, allows for the combined PET imaging. This highly specific \([^{64}\text{Cu}]\text{DOTA-mJF5}\) tracer allows repeated imaging and distinguishes aspergillosis from pulmonary inflammation and bacterial lung infections [73].

Only a year later, the same team reported the development of a humanized version of the JF5 antibody (hJF5). This new, \([^{64}\text{Cu}]\text{NODAGA-hJF5}\) tracer showed not only improved imaging capabilities but also a high specificity towards \( \text{Gal}(1\rightarrow3)\beta\text{-Gal} \) epitopes, present in a mannoprotein antigen released by Aspergillus during lung infection. This was the first time that Gal\( f \)-specific, antibody-guided in vivo imaging has been used for non-invasive preclinical diagnosis of a fungal lung disease [74].

These recent experimental techniques based on Gal\( f \) specific antibodies are still under development and are greatly contributing towards more specific targeting of epitope patterns. The related imaging and diagnostic aspects have yet to be explored.

5. Conclusions

The impact of modern biotechnology and recombinant DNA technology has made enzymes available in an economically feasible approach. It enabled a whole new diversity of enzymes to be accessed in the field of glycoenzymes. In parallel carbohydrate-based materials have emerged in an increasing number of applications in the food, feed, pharmaceutical and other industries. To access these carbohydrate structures that are in high demand, natural glycoenzyme catalysts have provided over the last few decades an alternative to chemical synthesis. A current area of interest is broadening the search spectrum to rare glycan substrate specific glycoside hydrolases, preferably, from narrow group of organisms involved in pathological conditions, in order to create altered characteristics, various functions and application possibilities within protein engineering. The biocatalyzed synthesis of galactofuranosyl containing conjugates still represents an emerging area due to our limited knowledge about the interaction of the protein with this specific and rare carbohydrate. Recent findings in the field described in this review render it very promising.

Author Contributions: All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Région Centre Val de Loire, APR IR Neolec, S.P. acknowledges support from Institut National de la Santé et de la Recherche Médicale (INSERM).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

| Abbreviation | Description                  |
|--------------|------------------------------|
| ELISA        | enzyme-linked immunosorbent assay |
| EPS          | exopolysaccharides           |
| EPS          | extracellular polysaccharides |
| FAD          | flavine adenine dinucleotide  |
| Gal\( f \)   | galactofuranose              |
| Gal\( T \)   | galactofuranosyltransferase  |
| Gal\( p \)   | galactopyranose              |
| GIPLs        | glycoinositolphospholipids   |
| GM           | galactomannan                |
HS toxin  host-selective toxin
LPGs  lipophosphoglycans
LPPG  lipopeptidophosphoglycan
LPS  lipopolysaccharide
ORF  open reading frame
PAMPs  pathogen-associated molecular patterns
PDB  Protein Data Bank
PET/MR  positron emission tomography/magnetic resonance
pNP-β-d-Galf  para-nitropheryl β-d-galactofuranose
pPGM  peptidophosphogalactomannan
UDP  uracil diphosphate
UDP-Galf  uracil diphosphate galactofuranose
UGM  UDP-galactopyranose mutase

References
1. Marino, C.; de Lederkremer, R.M. Galactose Configurations in Nature with Emphasis on the Biosynthesis of Galactofuranose in Glycans. In Galactose: Structure and Function in Biology and Medicine, 1st ed.; Pomin, V.H., Ed.; Nova Science Publisher Inc.: Hauppauge, NY, USA, 2014; Volume 2, pp. 107–133.
2. van Rantwijk, F. Monosaccharides. Their Chemistry and Their Roles in Natural Products. Rec. Trav. Chim. Pays-Bas 1996, 115, 420. [CrossRef]
3. Tefsøen, B.; Ram, A.F.; van Die, I.; Routier, F.H. Galactofuranose in Eukaryotes: Aspects of Biosynthesis and Functional Impact. Glycobiology 2012, 22, 456–469. [CrossRef]
4. Peltier, P.; Euzen, R.; Daniellou, R.; Nugier-Chauvin, C.; Ferrière, V. Recent Knowledge and Innovations Related to Hexofuranosides: Structure, Synthesis and Applications. Carbohydr. Res. 2008, 343, 1897–1923. [CrossRef] [PubMed]
5. Marino, C.; Gallo-Rodriguez, C.; de Lederkremer, R.M. Galactofuranosyl-Containing Glycans: Occurrence, Synthesis and Biochemistry. In Glycans: Biochemistry, Characterization and Applications, 1st ed.; Mora-Montes, H.M., Ed.; Nova Science Publisher Inc.: Hauppauge, NY, USA, 2012; pp. 207–268.
6. Eppe, G.; Bkassiny, S.E.; Vincent, S.P. Galactofuranose Biosynthesis: Discovery, Mechanisms and Therapeutic Relevance. In Carbohydrates in Drug Design and Discovery; Jiménez-Barbero, J., Cañada, F.J., Martin-Santamaria, S., Eds.; The Royal Society of Chemistry: Cambridge, UK, 2015; pp. 209–241. [CrossRef]
7. Haworth, W.N.; Porter, C.R. Isolation of Crystalline α- and β-Ethylglucofuranosides (γ-Ethylglucosides) and Other Crystalline Derivatives of Glucofuranose. J. Chem. Soc. Resumed 1929, 2796–2806. [CrossRef]
8. Clutterbuck, P.W.; Haworth, W.N.; Raistrick, H.; Smith, G.; Stacey, M. Studies in the Biochemistry of Micro-Organisms. Biochem. J. 1934, 28, 94–110. [CrossRef] [PubMed]
9. Haworth, W.N.; Raistrick, H.; Stacey, M. Polysaccharides Synthesised by Micro-Organisms. Biochem. J. 1937, 31, 640–644. [CrossRef]
10. Green, J.W.; Pacsu, E. Glycofuranosides and Thioglycofuranosides. III. New Crystalline Furanosides of d-Galactose and l-Arabinose. J. Am. Chem. Soc. 1938, 60, 2056–2057. [CrossRef]
11. Lowary, T.L. Twenty Years of Mycobacterial Glycans: Furanosides and Beyond. Acc. Chem. Res. 2016, 49, 1379–1388. [CrossRef]
12. Thanna, S.; Sucheck, S.J. Targeting the Trehalose Utilization Pathways of Mycobacterium Tuberculosis. MedChemComm 2016, 7, 69–85. [CrossRef]
13. Jankute, M.; Cox, J.A.G.; Harrison, J.; Besra, G.S. Assembly of the Mycobacterial Cell Wall. Annu. Rev. Microbiol. 2015, 69, 405–423. [CrossRef]
14. Brown, L.; Wolf, J.M.; Prados-Rosales, R.; Casadavall, A. Through the Wall: Extracellular Vesicles in Gram-Positive Bacteria. Mycobacteria and Fungi. Nat. Rev. Microbiol. 2015, 13, 620–630. [CrossRef] [PubMed]
15. Abrahams, K.A.; Besra, G.S. Mycobacterial Cell Wall Biosynthesis: A Multifaceted Antibiotic Target. Parasitology 2018, 145, 116–133. [CrossRef] [PubMed]
16. Nagaoka, M.; Hashimoto, S.; Shibata, H.; Kimura, I.; Kimura, K.; Sawada, H.; Yokokura, T. Structure of a Galactan from Cell Walls of Bifidobacterium Catenulatum YIT4016. Carbohydr. Res. 1996, 281, 285–291. [CrossRef]
17. Faber, E.J.; van den Haak, M.J.; Kamerling, J.P.; Vliegenthart, J.F.G. Structure of the Exopolysaccharide Produced by Streptococcus thermophilus S3. Carbohydr. Res. 2001, 331, 173–182. [CrossRef]
59. Mariño, K.; Lima, C.; Maldonado, S.; Marino, C.; de Lederkremer, R.M. Influence of Exo Beta-\(\alpha\)-Galactofuranosidase Inhibitors in Cultures of Penicillium fellutanum and Modifications in Hyphal Cell Structure. Carbohydr. Res. 2002, 337, 891–897. [CrossRef]

60. Dubourdieu, D.; Desplanches, C.; Villetaz, J.-C.; Ribereau-Gayon, P. Investigations of an Industrial beta-\(\alpha\)-Glucanase from Trichoderma harzianum. Carbohydr. Res. 1985, 144, 277–287. [CrossRef]

61. Marino, C.; Mariño, K.; Miletti, L.; Manso Alves, M.J.; Colli, W.; de Lederkremer, R.M. 1-Thio-beta-\(\alpha\)-Galactofuranosides: Synthesis and Evaluation as beta-\(\alpha\)-Galactofuranosidase Inhibitors. Glycobiology 1998, 8, 901–904. [CrossRef]

62. Varela, O.; Marino, C.; de Lederkremer, R.M. Synthesis of p-Nitrophenyl beta-\(\alpha\)-Galactofuranosidosede Lederkremer, R.M. Synthesis of p-Nitrophenyl beta-\(\alpha\)-Galactofuranoside. A Convenient Substrate for beta-\(\alpha\)-Galactofuranosidase. Carbohydr. Res. 1986, 155, 247–251. [CrossRef]

63. Matsunaga, E.; Higuchi, Y.; Mori, K.; Tashiro, K.; Kuhara, S.; Takegawa, K. Draft Genome Sequence of Streptomyces sp. JHA19, a Strain That Possesses beta-\(\alpha\)-Galactofuranosidase Activity. Genome Announc. 2015, 3, e01171-15. [CrossRef]

64. Matsunaga, E.; Higuchi, Y.; Mori, K.; Yairo, N.; Oka, T.; Shinozuka, S.; Tashiro, K.; Izumi, M.; Kuhara, S.; Takegawa, K. Identification and Characterization of a Novel Galactofuranose-Specific beta-\(\alpha\)-Galactofuranosidase from Streptomyces Species. PLOS ONE 2015, 10, e0137230. [CrossRef] [PubMed]

65. Matsunaga, E.; Higuchi, Y.; Mori, K.; Yairo, N.; Toyota, S.; Oka, T.; Tashiro, K.; Takegawa, K. Characterization of a PA14 Domain-Containing Galactofuranose-Specific beta-\(\alpha\)-Galactofuranosidase from Streptomyces sp. Biosci. Biotechnol. Biochem. 2017, 81, 1314–1319. [CrossRef] [PubMed]

66. Matsunaga, E.; Higuchi, Y.; Mori, K.; Tashiro, K.; Takegawa, K. Draft Genome Sequence of Streptomyces sp. JHA26, a Strain That Harbors a PA14 Domain Containing beta-\(\alpha\)-Galactofuranosidase. Genome Announc. 2017, 5, e00190-17. [CrossRef] [PubMed]

67. Seničar, M.; Legentil, L.; Ferrières, V.; Eliseeva, S.V.; Petoud, S.; Takegawa, K.; Lafite, P.; Daniellou, R. Galactofuranosidase from JHA 19 Streptomyces sp.: Subcloning and Biochemical Characterization. Carbohydr. Res. 2019, 480, 35–41. [CrossRef] [PubMed]

68. Bishop, J.R.; Gagneux, P. Evolution of Carbohydrate Antigens—Microbial Forces Shaping Host Glycomes? Glycobiology 2007, 17, 23–34. [CrossRef] [PubMed]

69. Heesemann, L.; Kotz, A.; Echtenacher, B.; Broniszewska, M.; Routier, F.; Hoffmann, P.; Ebel, F. Studies on Galactofuranose-Containing Glycostructures of the Pathogenic Mold Aspergillus fumigatus. Int. J. Med. Microbiol. 2011, 301, 523–530. [CrossRef]

70. Stynen, D.; Sarfati, J.; Goris, A.; Prévost, M.C.; Lesourd, M.; Kamphuis, H.; Darras, V.; Latgé, J.P. Rat Monoclonal Antibodies against Aspergillus Galactomannan. Infect. Immun. 1992, 60, 2237–2245. [CrossRef]

71. Verdaguer, V.; Walsh, T.J.; Hope, W.; Cortez, K.J. Galactomannan Antigen Detection in the Diagnosis of Invasive Aspergillusiosis. Expert Rev. Mol. Diagn. 2007, 7, 21–32. [CrossRef]

72. Marino, C.; Rinflerch, A.; de Lederkremer, R.M. Galactofuranose Antigens, a Target for Diagnosis of Fungal Infections in Humans. Future Sci. OA 2017, 3, FSO199. [CrossRef]

73. Rolle, A.-M.; Hasenberg, M.; Thornton, C.R.; Solouk-Saran, D.; Männ, L.; Weski, J.; Maurer, A.; Fischer, E.; Spycher, P.R.; Schibli, R.; et al. ImmunoPET/MR Imaging Allows Specific Detection of Aspergillus fumigatus Lung Infection In Vivo. Proc. Natl. Acad. Sci. USA 2016, 113, 1026–1033. [CrossRef]

74. Davies, G.; Rolle, A.-M.; Maurer, A.; Spycher, P.R.; Schilling, C.; Solouk-Saran, D.; Hasenberg, M.; Weski, J.; Fonslet, J.; Dubois, A.; et al. Towards Translational ImmunoPET/MR Imaging of Invasive Pulmonary Aspergillosis: The Humanised Monoclonal Antibody JF5 Detects Aspergillus Lung Infections In Vivo. Theranostics 2017, 7, 3398–3414. [CrossRef] [PubMed]