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The characterisation of a galactokinase from *Streptomyces coelicolor*

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Abstract

Promiscuous galactokinases (GalKs), which catalyse the ATP dependent phosphorylation of galactose in nature, have been widely exploited in biotechnology for the rapid synthesis of diverse sugar-1-phosphates. This work focuses on the characterisation of a bacterial GalK from *Streptomyces coelicolor* (ScGalK), which was overproduced in *Escherichia coli* and shown to phosphorylate galactose. ScGalK displayed a broad substrate tolerance, with activity towards Gal, GalN, Gal3D, GalNAc, Man and L-Ara. Most interestingly, ScGalK demonstrated a high activity over a broad pH and temperature range, suggesting that the enzyme could be highly amenable to multi-enzyme systems.

Key words: Galactokinase, Sugar-1-phosphates, *Streptomyces coelicolor*, Substrate specificity

1. Introduction

In nature, sugar phosphates have vital functions in physiological processes such as energy transfer and cell signalling, as well as being key constituents of nucleic acids [1, 2, 3, 4]. It is however, their roles in carbohydrate metabolism that have generated great interest in these compounds for pharmaceutical and biotechnological applications [5, 6, 7]. Sugar-1-phosphates in particular, are key intermediates in complex glycan biosynthesis, required for the formation of nucleotide-activated sugars [8].

Galactose-1-phosphate (Gal-1P, **2**) is an important intermediate in galactose (Gal, **1**) metabolism, which ultimately results in the biosynthesis of uridine diphosphate galactose (UDP-Gal, **3**). UDP-Gal (**3**) biosynthesis occurs by two major metabolic pathways, the salvage pathway and the Leloir pathway (**Fig. 1**) [9]. In the salvage pathway (**A**), a UDP-sugar pyrophosphorylase catalyses the conversion of Gal-1P (**2**) to UDP-Gal (**3**) in the presence of uridine triphosphate (UTP). In contrast, UDP-Gal (**3**) formation via the Leloir pathway (**B**) is catalysed by a galactose-1-phosphate uridylyltransferase and UDP-glucose-4-epimerase. UDP-Gal (**3**) is then used as a substrate by galactosyltransferases, that facilitate the incorporation of galactose into diverse glycan structures.

Various approaches exist for the synthesis of glycosyl phosphates (**2**). Chemical syntheses can be laborious, often requiring protecting group chemistry and can suffer from limited regio- and stereo-control [10, 11, 12, 13]. In contrast, enzymatic approaches often offer single-step syntheses, with high regio- and stereoselectivity, therefore eliminating the need for protecting group chemistry. Galactokinases (EC 2.7.1.6, GalKs), which catalyse the adenosine triphosphate (ATP) dependent phosphorylation of galactose in nature (**Fig. 1**), have been widely exploited in biotechnology for their ability to synthesise diverse sugar-1-phosphates for natural product diversification [14, 15]. GalKs belong to the GHMP family, that includes homoserine kinases, mevalonate kinases and

phosphomevalonate kinases [16]. While many eukaryotic GalKs have been characterised and studied for their role in type II galactosemia [17, 18, 19, 20, 21, 22, 23], it has been the characterisation of bacterial GalKs with their broad substrate preferences and high expression levels, that have really proven to be of great value in biotechnology [24, 25, 26, 27, 28]. In particular, GalKs have been widely exploited in multi enzyme systems for the chemo enzymatic synthesis of sugar nucleotides [29, 30] and β 1,3 galactosides [31, 32].

Herein, we describe the cloning, overexpression and characterisation of the galactokinase from the soil bacterium, *Streptomyces coelicolor*. Investigation of its biochemical properties revealed the enzyme to maintain high activity over a broad pH and temperature range, suggesting that the enzyme could be highly amenable to one-pot multi enzyme systems. Additionally, studies of substrate specificity have revealed the enzyme to be active towards a range of unnatural substrates, demonstrating its potential for biotechnological exploitation.

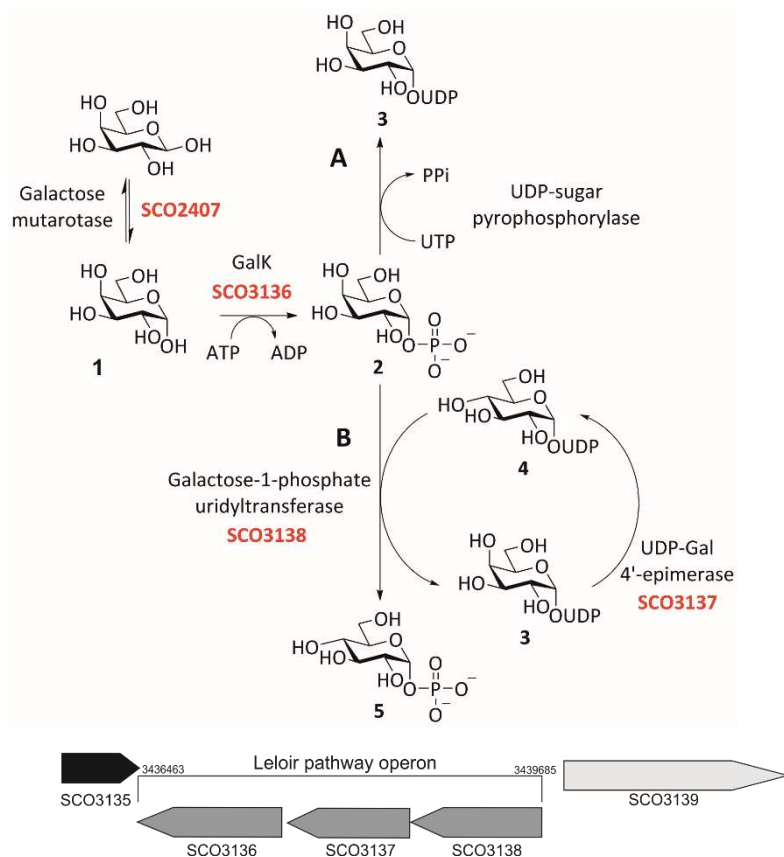


Fig. 1 GalK mediated synthesis of Gal-1P (2) is an important precursor for UDP-Gal production via the salvage pathway (A) and the Leloir pathway (B). The SCO numbers for the proteins encoded in this pathway in *S. coelicolor* are shown. *sco3136* (ScGalK) in *S. coelicolor* is found in the Leloir pathway operon that also encodes SCO3137 (GalE) and SCO3138 (GalT). Image to reproduced in colour on the Web (free of charge) and in black-and-white in print.

2. Results and Discussion:

2.1 Cloning, over-expression and purification of ScGalK

The GalK from *S. coelicolor* (ScGalK) was previously investigated as a reporter for *in vivo* promoter strength [33], however the enzyme has never been heterologously expressed and its substrate specificity is currently unexplored. The gene encoding ScGalK in the *S. coelicolor* genome, *sco3136*, forms part of the Leloir pathway operon (**Fig. 1**), which also encodes a putative UDP-Gal 4-epimerase (SCO3137) and a galactose-1-phosphate uridylyltransferase (SCO3138). An alignment of the ScGalK amino acid sequence with the sequences of previously characterised GalKs from *Escherichia coli* (*E. coli*, EcGalK), *Lactococcus lactis* (*L. lactis*, LIGalK), *Streptococcus pneumoniae* (*S. pneumoniae*, GalKSpe4), *Leminorella grimontii* (*L. grimontii*, LgGalK), *Bifidobacterium infantis* (*B. infantis*, BiGalK) and *Meiothermus taiwanensis* (*M. taiwanensis*, MtGalK) demonstrated that the three characteristic GHMP superfamily motifs were also conserved in ScGalK (**Fig. S1**) [34]. Additionally, ScGalK contains the two highly conserved catalytic amino acid residues, R30 and D191, that were previously demonstrated to be required for GalK function in *L. lactis* [35]. ScGalK was amplified by PCR from *S. coelicolor* A3(2) J1929 genomic DNA and cloned into pET15b with an N-terminal His₆-tag. ScGalK was overexpressed in *E. coli* BL21(DE3) under induction of 0.8 mM isopropyl-1-thio- β -D-galactopyranoside IPTG (16 °C for 22 h). The recombinant protein was purified by using a two-step purification procedure, first by Ni²⁺ affinity chromatography and then by gel filtration chromatography. A typical yield of active recombinant ScGalK was \approx 33 mg/L of cell culture. The mobility of recombinant ScGalK by SDS-PAGE was consistent with the predicted molecular mass of the protein of 43.1 kDa (**Fig. 2**).

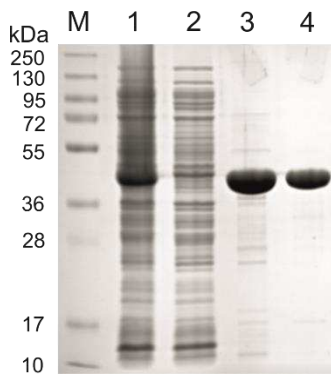


Fig. 2 SDS-PAGE analysis of ScGalK. Lanes: (M) protein Mw standard, (1) Total soluble, (2) Flow through, (3) Elution Ni²⁺ affinity chromatography, (4) Elution - size exclusion chromatography

2.2 Biochemical characterisation of ScGalK

To determine the biochemical properties of ScGalK, the effects of temperature, pH and metal ions were investigated on the activity of the enzyme for its natural substrate, Gal (1) (**Fig. 3**). The optimum temperature was found to be at 45 °C, with activity rapidly decreasing at higher temperatures (**Fig. 3A**). Interestingly, ScGalK appeared to retain a relatively high activity at lower temperatures, displaying $\approx 60\%$ activity at 4 °C. In contrast to many of the previously characterised bacterial GalKs that display significant decreases in activity at lower temperatures, including GalKSpe4 ($\approx 45\%$ activity at 25 °C), LgGalK ($\approx 40\%$ activity at 4 °C) and BiGalK ($\approx 15\%$ activity at 4 °C), this is quite an unusual characteristic [25, 26, 27]. ScGalK also sustains its activity over a wide pH range, with $> 80\%$ activity observed in the pH range 4 – 10 (**Fig. 3B**). This feature is most similar to GalKSpe4, which also maintains $> 80\%$ activity over the same pH range [25]. In contrast, maximal activity was observed only in the pH ranges 7.5 – 9 for BiGalK and 8 – 10 in LgGalK [26, 27].

As observed in all previously characterised bacterial GalKs to date [24, 25, 26, 27, 28], ScGalK was shown to require Mg^{2+} as a cofactor (**Fig. 3C**). Amongst the bivalent metal cations tested, Zn^{2+} could partially substitute for Mg^{2+} . In contrast, ScGalK activity was nearly eliminated in the presence of Ca^{2+} , Mn^{2+} and Cu^{2+} .

The kinetic parameters of ScGalK towards Gal (**1**) and ATP were determined by coupling the formation of adenosine diphosphate (ADP) to the reactions catalysed by pyruvate kinase and lactate dehydrogenase [36] (**Table 1**). Reactions were carried out under saturating concentrations of ATP (10 mM) with different concentrations of Gal (**1**), and with different concentrations of ATP under saturating conditions of Gal (**1**) respectively. The initial velocity was determined from the gradient of the linear phase of the reaction progress curve, and Michaelis-Menten plots were generated to obtain the apparent kinetic parameters for each substrate (**Fig. S2**). The K_m values of ScGalK towards Gal (**1**) (0.627 mM) were comparable to those of BiGalK (0.895 mM), EcGalK (2.1 mM) and LgGalK (1.3 mM), however the enzyme displayed a significantly higher affinity for Gal (**1**) than GalKSpe4 (37.23 mM) [24, 25, 26, 27]. The catalytic efficiency of the enzyme for Gal (**1**) ($2.15\text{ s}^{-1}\text{ mM}^{-1}$) was higher than with GalKSpe4 ($0.07\text{ s}^{-1}\text{ mM}^{-1}$) and EcGalK ($0.552\text{ s}^{-1}\text{ mM}^{-1}$) [24, 25]. In contrast, higher catalytic efficiencies towards Gal (**1**) have been observed previously by BiGalK ($164\text{ s}^{-1}\text{ mM}^{-1}$) and LgGalK ($18.1\text{ s}^{-1}\text{ mM}^{-1}$) [26, 27]. Overall, the kinetic parameters of ScGalK show that the enzyme bears similarity to EcGalK.

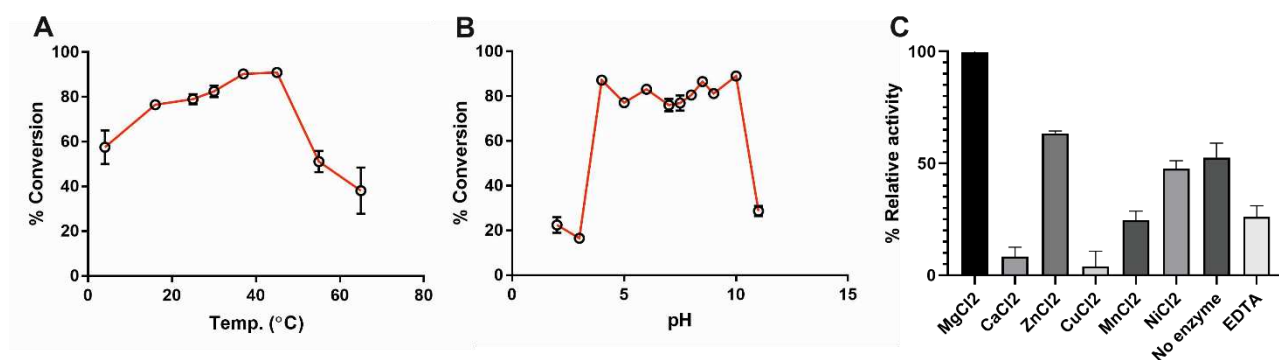


Fig. 3 Physicochemical properties of ScGalk. **(A)** Temperature-activity profile. **(B)** pH- activity profile. **(C)** Metal ion dependence. Data represent the mean of three replicates. Error bars represent the standard error of the mean. Image to be reproduced in colour on the Web (free of charge) and in black-and-white in print.

Table 1. Kinetic parameters for ScGalk

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} mM^{-1}$)
Gal	0.627 ± 0.061	1.350 ± 0.044	2.15
ATP	0.035 ± 0.004	1.350 ± 0.033	38.90

2.3 Determination of ScGalk substrate specificity

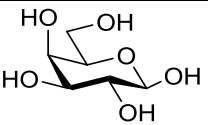
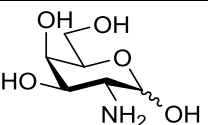
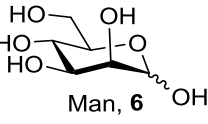
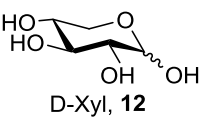
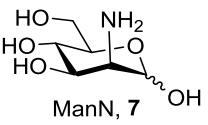
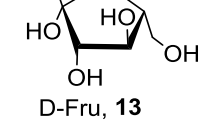
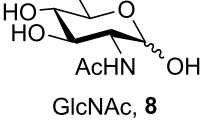
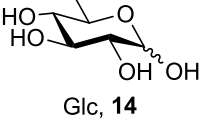
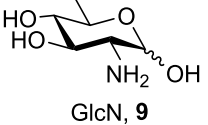
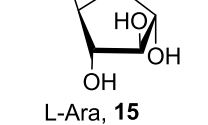
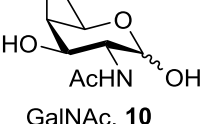
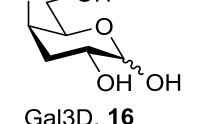
In the initial studies of ScGalk substrate specificity, reactions were screened by TLC (**Figure S3**) and the presence of the glycosyl-1-phosphate products were validated by high resolution mass spectrometry (HRMS). The conversions of the substrates identified as hits during the initial screens were then determined by using the dinitrosalicylic acid (DNS) assay (**Table 2**) [24]. As expected, ScGalk demonstrated a high specificity for its natural substrate Gal (**1**), enabling 91% conversion after 2 h under optimised conditions. To further probe the substrate specificity of ScGalk and its potential for use in biocatalysis, 11 different monosaccharide substrates were investigated (**Table 2**). Reactions that were carried out with the unnatural substrates, were allowed to proceed for 24 h

and contained \approx 4-fold more enzyme than the reactions with Gal (**1**). We anticipated that the increased reaction times and enzyme concentrations might help to identify substrates that, due to their reduced affinity or catalytic turnover by the enzyme, may not otherwise be observed as substrates. ScGalK was found to have some activity towards 8 out of the 11 unnatural substrates that were tested – mannose (**6**), mannosamine (**7**), N-acetyl-D-glucosamine (**8**), N-acetyl-D-galactosamine (**10**), galactosamine (**11**), glucose (**14**), L-arabinose (**15**) and 3-deoxy-galactose (**16**). As shown in **Table 2**, the galactose derivatives **10** and **11** were observed as substrates for the enzyme, displaying conversions of 40% and 87% respectively. The lower conversion of **10** by comparison to **11** is consistent with previous observations that GalKs can tolerate small modifications to the C-2 position, however groups with increased steric bulk result in little or no substrate specificity [24, 25, 26, 27, 28]. ScGalK displayed high specificity towards **16**, suggesting that the removal of the C-3 hydroxyl was well tolerated by the enzyme. Gluco-configured substrates were not well tolerated by the enzyme. This is consistent with observations made previously in other bacterial GalKs [24, 26, 27, 28]. Interestingly, ScGalK did show some activity towards D-mannose (**6**). The only previous report of galactokinase activity towards mannose was in the human galactokinase (GalK1) Y379C and Y379W mutants [37]. Timson and Kristiansson demonstrated that mutagenesis of residue Y379 in GalK1 resulted in an enzyme with broadened substrate specificity. While the Y379 residue is not located within the GalK1 active site, amino acid changes at this position were shown to increase the overall flexibility of the enzyme. Interestingly, alterations to the equivalent residues in EcGalK (Y371) and LIGalK (Y385) have also been shown to increase enzyme substrate specificity [5, 6, 38]. Based on the amino acid sequence alignment of the characterised bacterial GalKs (**Fig. S1**), a conserved tyrosine residue that aligns with Y371 in EcGalK and Y385 in LIGalK, was observed in LgGalK and GalKSpe4. In contrast, ScGalK appears to lack a tyrosine at this position, which might contribute towards the broad substrate range of the enzyme. Interestingly, ScGalK also

demonstrated some low-level activity towards L-arabinose (**15**), which has only ever been reported in the wild-type GalK from *Phaseolus aureus* [39].

The broad substrate tolerance of ScGalK, as well as the fact that the enzyme maintains high-level activity over a wide pH and temperature range (**Fig. 3**), suggests that the enzyme could be used in one-pot multi enzyme cascades for the chemo enzymatic synthesis of carbohydrates. For example, the unique activity of ScGalK towards L-arabinose could be exploited for the synthesis of UDP-L-arabinose, by coupling the enzyme with the UDP-sugar pyrophosphorylase from *Pisum sativum* L., that was previously shown to use L-arabinose-1-phosphate as a substrate [40]. Additionally, the activity of ScGalK towards mannose suggests that the enzyme could be utilised as an alternative to an *N*-acetylhexosamine-1-kinase (NahK), in the one-pot multi enzyme synthesis of guanosine diphosphate (GDP) and uridine diphosphate (UDP) mannose, in reactions coupled with GDP- and UDP-sugar pyrophosphorylases and inorganic pyrophosphatases [29, 41].

Table 2. Substrate specificity of ScGalK towards a panel of monosaccharides

Substrate	HRMS ^(a)	TLC ^(a)	Conv. (%)	Substrate	HRMS ^(a)	TLC ^(a)	Conv. (%)
 Gal, 1	+	+	91	 GalN, 11	+	+	87
 Man, 6	+	+	21	 D-Xyl, 12	-	-	<i>n.d.</i>
 ManN, 7	+	-	<i>n.d.</i>	 D-Fru, 13	-	-	<i>n.d.</i>
 GlcNAc, 8	+	+	< 5	 Glc, 14	+	-	6
 GlcN, 9	-	-	<i>n.d.</i>	 L-Ara, 15	+	+	10
 GalNAc, 10	+	+	40	 Gal3D, 16	+	+	85

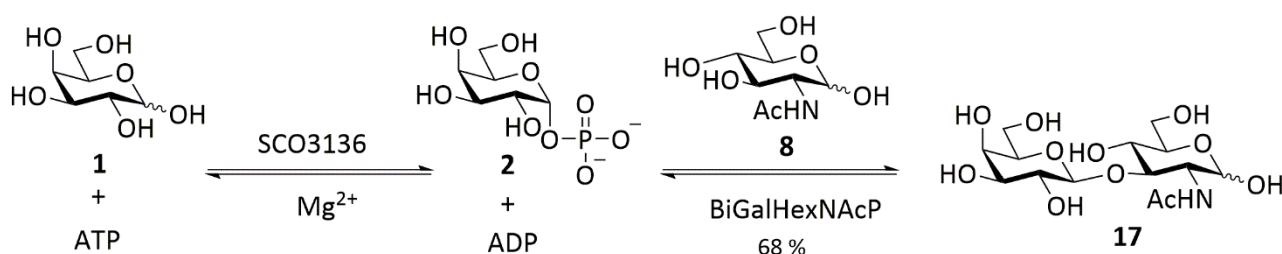
(a) +: product observed, -: no product observed. *n.d.* : – conversion not determined; HRMS =

High resolution mass spectrometry; TLC = thin layer chromatography.

2.4 Use of SCO3138 in a one-pot two enzyme synthesis of lacto-n-biose.

To demonstrate the utility of ScGalK, the enzyme was used in a one-pot two enzyme system (**Scheme 1**) with the D-galactosyl- β 1–3-*N*-acetyl-D-hexosamine phosphorylase from *B. infantis* (BiGalHexNAcP) for the synthesis of lacto-n-biose. BiGalHexNAcP catalyses the phosphorolysis of lacto-n-biose and galacto-n-biose, to produce Gal-1-P (**2**) and the corresponding N-acetyl-D-

hexosamine. However, by coupling BiGalHexNAcP with GalKs in a one-pot system, it is possible to utilise the enzyme's reverse reaction for the synthesis of β 1-3 galactosides [31, 32, 42]. BiGalHexNAcP has a relatively narrow pH range (5 – 6.5) under which it displays maximal activity [31]. Therefore, reactions were carried out at pH 6.5 to ascertain that both ScGalK and BiGalHexNAcP displayed high activity. For the synthesis of lacto-n-biose (**17**), reactions were assembled on a 50 mg scale with Gal (**1**), GlcNAc (**8**), ScGalK and BiGalHexNAcP in the presence of Mg^{2+} and ATP. After proceeding for 48 h, the reaction products were purified by anion exchange and then gel filtration chromatography, and the synthesis of lacto-n-biose (**17**) was validated by NMR (**Figure S4-8**). Lacto-n-biose was isolated in a moderate yield of 68%, which is lower than the 95% yield previously reported by Xi Chen and co-workers using the EcGalK/BiGalHexNAcP coupled system [31]. However, as the reaction appeared to proceed to completion (**Fig. S10**), the lower yield could be explained by the smaller reaction scale and differences in purification methodologies.



Scheme 1: One-pot two enzyme synthesis of lacto-n-biose (**4**) catalysed by ScGalK and BiGalHexNAcP.

3. Conclusions

We have characterised and overexpressed a new bacterial galactokinase (SCO3138) from *Streptomyces coelicolor*. We have investigated the effects of temperature, pH and metal ions to determine the optimal biochemical conditions of the enzyme: 45 °C, pH 4 - 10, Mg²⁺. ScGalK demonstrated a high activity over a broad temperature and pH range, suggesting that the enzyme could be highly amenable to multi-enzyme systems. This was demonstrated by coupling the enzyme in one pot with BiGalHexNAcP for the synthesis of lacto-n-biose. Kinetic characterisation of the enzyme for its natural substrates afforded the kinetic constants for Gal: $K_m = 0.627 \text{ mM} \pm 0.061$, $K_{cat}/K_m = 2.15 \text{ s}^{-1} \text{ mM}^{-1}$ and ATP: $K_m = 0.035 \text{ mM} \pm 0.004$, $K_{cat}/K_m = 38.90 \text{ s}^{-1} \text{ mM}^{-1}$. ScGalK demonstrated a broad substrate specificity, turning over substrates such as GalN, Gal3D and GalNAc and also displayed activity towards Man and L-Ara, which has never been reported in a wild type bacterial GalK.

4. Experimental

4.1 General procedures

All chemical reagents and solvents were purchased from commercial sources and used without further purification, unless stated otherwise. Samples for HRMS data analysis were submitted to the University of York Centre of Excellence in Mass Spectrometry (CoEMS) and analysed at room temperature on a Bruker Daltonics microTOF spectrometer. Reactions were analysed by silica gel thin-layer chromatography (TLC) by spotting 0.5 μL of each sample onto a TLC plate. A solvent system of nBuOH/AcOH/H₂O (2:1:1) was used and TLC plates were stained with p-anisaldehyde. ¹H and ¹³C NMR spectra were acquired on a Bruker 500-MR spectrometer at 500 MHz for ¹H and 125 MHz for ¹³C. Multiplicities are given as singlet (s), doublet (d), doublet of doublets (dd), triplet of doublets (td), or multiplet (m). Resonances were assigned using HH-COSY and CHHMQC. All NMR

chemical shifts (δ) were recorded in ppm and coupling constants (J) are reported in Hz. TopSpin 3.5pl7 and MestReNova were primarily used for processing the spectral data.

4.2 Molecular cloning

sco3136 (ScGalk) was amplified by PCR from *Streptomyces coelicolor* A3(2) J1929 [43] genomic DNA, using primers TK19 (5'-CGCGCGGCAGCCATATGATGGGCGAGGCTGTCGCGGG AA-3') and TK20 (5'-GTTAGCAGCCGGATCCTCAGACCAGGCGCCGCGCCCCG-3'). The PCR product was purified by gel extraction using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), and cloned into NdeI and BamHI digested pET15b using the Quick-Fusion Cloning Kit (bioutil). The resulting construct, SCO3136_pET15b was introduced into chemically competent *E. coli* DH5 α cells by heat shock and the transformants were selected on lysogeny broth (LB) agar supplemented with ampicillin (100 μ g/mL). Plasmid DNA was isolated by using the QIAprep Spin Miniprep Kit (QIAGEN) and the SCO3136_pET15b construct was verified by DNA sequencing.

4.3 Protein production and purification

The SCO3136_pET15b plasmid was introduced into chemically competent *E. coli* BL21(DE3) cells by heat shock and selected on LB agar with ampicillin (100 μ g/mL) at 37 °C for 16 h. Starter cultures were prepared by picking single clones into LB with ampicillin (100 μ g/mL) and grown at 37 °C for 6 h with shaking (180 rpm). 10 mL of starter culture was added to 1L of LB with ampicillin (100 μ g/mL) and grown to an OD₆₀₀ of 0.8 at 37 °C with shaking (180 rpm). Cultures were placed in an ice bath for 10 min. IPTG was added to a final concentration of 0.8 mM and the cultures were grown at 16 °C for 22 h with shaking (180 rpm). Cells were harvested by centrifugation (6000 x g, 6 °C, 10 min)

and the pellet was resuspended in lysis buffer (30 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mg/mL lysozyme, protease inhibitor, benzonase 1 U/mL). The cells were lysed by sonication on ice and the lysate was clarified by centrifugation (18 000 rpm, 4 °C, 45 min). The lysate was loaded onto a 5 mL HisTrap FF column (GE Healthcare) pre-equilibrated with binding buffer (30 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole). After washing the column with 20 CV of binding buffer, recombinant ScGalK with an N-terminal His₆-tag was eluted with elution buffer (30 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole). The protein was desalted and exchanged into 30 mM Tris pH 8.0 buffer using HiPrep™ 26/10 Desalting column (GE Healthcare). Further purification was carried out by using gel filtration chromatography (HiLoad 16/600 Superdex 200 pg) in 30 mM Tris pH 8.0 buffer. Typical yield was \approx 33 mg/L culture. Purified ScGalK was stored at -80 °C in 10% (v/v) glycerol.

4.4 Determination of substrate specificity

Reactions were typically carried out in triplicate, on a 60 μ L scale. Reactions were carried out in Tris buffer (60 μ L, 100 mM, pH 8.0) at 37 °C for 2 h (substrate **1**) or 18 - 24 h (substrates **2** - **12**). For substrate **1**, specificity was determined in reactions containing monosaccharide (8 mM), ATP (10 mM), MgCl₂ (5 mM) and ScGalK (0.12 μ g/ μ L). For substrates **2** - **12**, specificity was determined in reactions containing monosaccharide (8 mM), ATP (10 mM), MgCl₂ (5 mM) and ScGalK (0.5 μ g/ μ L). The presence of the sugar-1-phosphate product was determined by HRMS and TLC. The conversion of monosaccharide substrates was determined by using the DNS assay, as described previously by [24]. Briefly, 50 μ L of sample was added to 100 μ L of DNS reagent. Samples were heated to 100 °C for 15 min, 4 °C for 1 min and 20 °C for 3 min in a PCR thermocycler, 15 μ L of 40% (w/v) potassium tartrate solution was added to each sample and the absorbance at 575_{nm} was recorded. Standard curves were prepared by using a series of sugar concentrations (0.5, 1, 2, 2.5, 3, 3.5, 4, 5, 6, 7, and

8 mM respectively) and ATP (10 mM) in Tris buffer (100 mM, pH 8.0) and submitting them to the DNS assay.

4.5 Influence of temperature and pH

For the temperature assay, reactions were prepared in triplicate on a 60 μ L scale with substrate **1** (8 mM), ATP (10 mM), $MgCl_2$ (5 mM) and Tris buffer (100 mM, pH 8.0). Reactions were pre-incubated at 4, 16, 25, 30, 37, 45, 55 and 65 $^{\circ}C$ respectively for 5 min. ScGalk (7.2 μ g) was added and the reactions were further incubated at the respective temperatures for 20 min. Substrate conversions were determined by the DNS assay.

For the pH assay, reactions were prepared identically to the temperature assay with different buffers varying in pH (pH 2, 3 - glycine-HCl, pH 4, 5, 6 - sodium acetate, pH 7 - HEPES, pH 7.5, 8, 8.5 - Tris-HCl and pH 9, 10 and 11 - glycine-NaOH). The reactions were pre-incubated at 37 $^{\circ}C$ for 5 min before the addition of ScGalk (7.2 μ g). The reactions were further incubated at 37 $^{\circ}C$ for 20 min and the conversion of substrate **1** was determined via the DNS assay.

For the metal ion screen, reactions were prepared in triplicate on a 60 μ L scale with substrate **1** (8 mM), ATP (10 mM), Tris buffer (100 mM, pH 8.0) and different metal ions (Mg^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} , Mn^{2+} or Ni^{2+} at 5 mM) or EDTA (5 mM). The reactions were pre-incubated at 37 $^{\circ}C$ for 5 min before the addition of ScGalk (7.2 μ g). The reactions were further incubated at 37 $^{\circ}C$ for 20 min and the conversion of substrate **1** was determined via the DNS assay.

2.6 Determination of ScGalk kinetic parameters

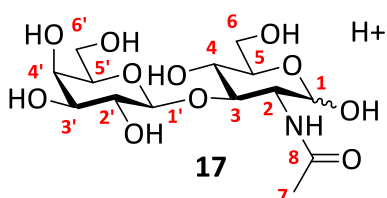
Galactokinase activity was determined by coupling the formation of ADP to the reactions catalysed by pyruvate kinase and lactate dehydrogenase and the consumption of NADH, as previously described [36]. Reactions (150 μ L) were assembled in microtitre plates containing 50 mM HEPES buffer (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM phosphoenol pyruvate, 1 mM NADH, 7.5 U pyruvate kinase and 10 U lactate dehydrogenase. Reactions were supplemented with various concentrations of galactose (0.0625 - 4 mM) under saturating concentrations of ATP (10 mM), or with various concentrations of ATP (0.02 – 1.5 mM) under saturating concentrations of Gal (10 mM). All reaction components were added to the microtitre plate except for ScGalK, mixed and preincubated at 37 °C for 5 min. Reactions were initiated by the addition of ScGalK (161 nM) and the decrease in absorbance at 340 nm was measured every 30 s for 5 min. The initial velocity was calculated by determining the linear phase in the progress curve. Assays were assembled in triplicate. The data were fitted to the Michaelis-Menten equation and the apparent kinetic parameters were calculated by using GraphPad Prism v. 7.05.

4.7 BiGalHexNAcP expression and purification

The BiGalHexNAcP expression conditions were based on the procedure previously reported by Xi Chen and colleagues [31]. The BiGalHexNAcP_pET15b plasmid was introduced into chemically competent *E. coli* BL21(DE3) cells by heat shock and selected on LB agar with ampicillin (100 μ g/mL) at 37 °C for 16 h. Starter cultures were prepared by picking single clones into LB with ampicillin (100 μ g/mL) and grown at 37 °C for 6 h with shaking (180 rpm). 10 mL of starter culture was added to 1L of LB with ampicillin (100 μ g/mL) and grown to an OD₆₀₀ of 0.8 at 37 °C with shaking (180 rpm). IPTG was added to a final concentration of 0.1 mM and the cultures were grown at 25 °C for 22 h with shaking (180 rpm). Cells were harvested by centrifugation (6000 x g, 6 °C, 10 min) and the pellet was

resuspended in lysis buffer (30 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mg/mL lysozyme, protease inhibitor, benzonase 1 U/mL). The cells were lysed by sonication on ice and the lysate was clarified by centrifugation (18 000 rpm, 4 °C, 45 min). The lysate was loaded onto a 5 mL HisTrap FF column (GE Healthcare) pre-equilibrated with binding buffer (30 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole). After washing the column with 20 CV of binding buffer, recombinant BiGalHexNAcP with an N-terminal His₆-tag was eluted with elution buffer (30 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole). Fractions containing BiGalHexNAcP were identified by 12% SDS-PAGE (**Fig. S9**) and pooled. The expected Mw of the protein was 86.5 kDa. The protein was dialysed in dialysis buffer (30 mM Tris pH 8.0) at 4 °C for 20 h, replacing with fresh dialysis buffer after 16 h. A yield of ~102 mg L⁻¹ of cell culture was retrieved. Purified BiGalHexNAcP was stored at -80 °C in 10% (v/v) glycerol.

4.8 Large scale one-pot two enzyme (1P2E) synthesis of lacto-*N*-biose (**17**)



The 1P2E synthesis of lacto-*n*-biose (**17**) was demonstrated on a 50 mg scale. The reaction was prepared by the addition of GlcNAc (**8**) (15 mM), Gal (**1**) (24 mM), ATP (15 mM), MgCl₂ (20 mM) and Tris buffer (100 mM, pH 6.5) and adjusted to pH 6.5. ScGalK (45 mg) and BiGalHexNAcP (33 mg) were added and the reaction was incubated at 37 °C for 48 h. TLC was used to validate the depletion of GlcNAc (**8**) (**Fig. S10**). The enzyme was removed from the reaction mixture using a Vivaspin centrifugal concentrator (30 kDa MwCo). The reaction was diluted 10-fold in H₂O and applied to a HiTrap Q HP anion exchange chromatography column (GE Healthcare) for the removal of the unreacted Gal-1P (**2**) and ADP. The column was washed with 5 CV of H₂O. The disaccharide

containing flow-through and wash fractions were retained and lyophilised. The Gal-1P (**2**) and ADP were eluted from the column by using a 0-100 % gradient of 1 M NH_4HCO_3 and discarded. The disaccharide containing fraction was resolubilised in water and purified by gel filtration chromatography using a Bio-Gel P-2 polyacrylamide column (Bio-Rad) in H_2O . Disaccharide containing fractions were pooled, lyophilised and validated by NMR (**Fig. S4- S8**). The product **17** was obtained as a 60:40 mixture of α : β anomers.

Major = C-1 α , Minor = C-1 β

β -D-Galactopyranosyl-(1 \rightarrow 3)-2-acetamido-2-deoxy-D-glucopyranose (Gal β 1 \rightarrow 3GlcNAc, **21**). Yield, 68%; white powder. ^1H NMR (500 MHz, D_2O) δ 5.20 (d, J = 3.5 Hz, 0.6H, **H-1 major**), 4.77 (d, J = 8.5 Hz, 0.4 H, **H-1 minor**), 4.49 (d, J = 7.7 Hz, 0.6H, **H-1' major**), 4.45 (d, J = 7.7 Hz, 0.4H, **H-1 minor**), 4.09 (dd, 0.6 H, J = 10.6, 3.6 Hz, **H-2 major**), 3.98 – 3.77 (m, 7H, **H-3, H-4', H-5, H-6, H-6' major; H-2, H-3, H-4', H-6, H-6' minor**), 3.74 (m, J = 6.4, 3.2, 1.4 Hz, 1H, **H-3' major & minor**), 3.67 (dt, J = 9.9, 3.4 Hz, 1H, **H-5' major & minor**), 3.64 – 3.50 (m, 2.4H, **H-2', H-4 major; H-2', H-4, H-5 minor**), 2.06 (s, 3H, **NHCOCH $_3$ major & minor**). ^{13}C NMR (125 MHz, D_2O) δ 174.73 & 174.47 (**NHCOCH $_3$ major & minor**), 103.49 (**C-1' minor**), 103.37 (**C-1' major**), 94.65 (**C-1 minor**), 90.96 (**C-1 major**), 82.55, 80.11, 75.39, 75.21, 75.16, 72.49, 72.45, 71.16, 70.66, 70.61, 68.64, 68.61, 68.48, 68.46, 60.93, 60.67, 60.51, 59.34, 55.56, 52.82, 22.18 & 21.93 (**NHCOCH $_3$ major & minor**). HRMS (ESI) m/z calcd for $\text{C}_{14}\text{H}_{26}\text{NO}_{11}$ (M + H) 384.1500, found 384.1503

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