

1 **Structural dissection of the active site of *Thermotoga***
2 ***marítima* β -galactosidase identifies key residues for**
3 **transglycosylating activity**

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5 **David Talens-Perales, Julio Polaina and Julia Marín-Navarro***

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7 Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Paterna, Valencia, Spain.

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12 *Corresponding author.

13 *E-mail address:* jumana@iata.csic.es (J. Marín-Navarro)

14

15 **ABSTRACT**

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17 Glycoside hydrolases, specifically β -galactosidases, can be used to synthesize
18 galacto-oligosaccharides (GOS) due to the transglycosylating (secondary) activity
19 of these enzymes. Site-directed mutagenesis of a thermoresistant β -galactosidase
20 from *Thermotoga maritima* has been carried out to study the structural basis of
21 transgalactosylation and to obtain enzymatic variants with better performance for
22 GOS biosynthesis. Rational design of mutations was based on homologous
23 sequence analysis and structural modelling. Analysis of mutant enzymes indicated
24 that residue W959, or an alternative aromatic residue at this position, is critical for
25 the synthesis of β -3'-galactosyl-lactose, the major GOS obtained with the wild-type
26 enzyme. Mutants W959A and W959C, but not W959F, showed an 80% reduced
27 synthesis of this GOS. Other substitutions: N574S, N574A and F571L increased the
28 synthesis of β -3'-galactosyl-lactose about 40%. Double mutants F571L/N574S and
29 F571L/N574A showed an increase of about twofold.

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32 **KEYWORDS:** enzyme engineering, galacto-oligosaccharides, GH2 glycoside
33 hydrolase, prebiotics

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36 **INTRODUCTION**

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38 Consumer demand of prebiotic compounds has risen sharply in the past
39 years. Consequently, the development of methods for the synthesis of these
40 products has become an industrially relevant target. Galacto-oligosaccharides
41 (GOS) constitute one of the major types of prebiotic molecules. They consist of
42 short chains (2 to 10) of galactose residues linked in some cases to a terminal
43 glucose at the reducing end. GOS are resistant to gastric acid and digestive
44 enzymes. When they reach the colon, they are preferentially metabolised by
45 beneficial intestinal bacteria (prebiotic effect)¹. GOS have been associated to
46 multiple health-promoting effects, such as hindering enteropathogens adhesion to
47 intestinal epithelium², improving calcium absorption and preventing
48 cardiovascular pathologies³. Chemically, GOS differ in their polymerization degree
49 and in the type of linkage that connects the galactosyl units (β -(1-2), β -(1-3), β -(1-
50 4) or β -(1-6))⁴. These differences have been related to their stability and prebiotic
51 potential^{5,6}.

52 GOS are produced by transgalactosylation of lactose by β -galactosidases.
53 According to the carbohydrate active enzyme database (Cazy), β -galactosidases are
54 found in four related families (GH1, GH2, GH35 and GH42) grouped in the GH-A
55 clan of glycoside hydrolases⁷. A common structural feature of the proteins of this
56 clan is the presence of a TIM barrel, defined by eight parallel beta strands
57 surrounded by eight α -helices, harbouring the catalytic residues^{7,8}. These enzymes
58 proceed through a two-step retaining mechanism that enables them to catalyse
59 both hydrolysis and transglycosylation^{9,10} (Supplementary Figure S1). In the first

60 step, the catalytic residue acting as nucleophile forms a covalent intermediate with
61 the galactosyl moiety from lactose, and the acid/base catalyst assists the departure
62 of the glucosyl unit. In the second step, the galactosyl group is transferred to an
63 acceptor deprotonated by the acid/base catalyst. This acceptor can be either a
64 water molecule or another lactose molecule, resulting in final hydrolysis or
65 transgalactosylation, respectively⁵. Different retaining glycosyl hydrolases have
66 been effectively used to synthesize functional oligosaccharides. For instance,
67 modified versions of yeast invertase that produce high yields of 6-kestotriose have
68 been obtained^{11,12}.

69 High temperature is advantageous for large-scale GOS synthesis since it
70 reduces the risk of microbial contamination and increases lactose solubility, which
71 in turn enhances GOS yield¹³. To work under these conditions, thermostable
72 enzymes are required. Some examples of thermoresistant β -galactosidases with
73 transgalactosylating capacity are found in families GH1 (from *Sulfolobus*
74 *solfataricus*, *Pirococcus furiosus* and *Thermus sp.*) and GH2 (from *Streptococcus*
75 *thermophilus* and *Thermotoga maritima*), showing different product specificity and
76 yields¹⁴⁻¹⁷.

77 In this work we have analysed structural and functional properties of a
78 heat-stable GH2 β -galactosidase from the thermophilic bacterium *Thermotoga*
79 *maritima*. This enzyme (henceforth TmLac) produces β -3'-galactosyl-lactose and β -
80 6'-galactosyl-lactose^{18,19}. We have investigated the role of different residues
81 located at the catalytic centre in the transgalactosylation reaction. Our study
82 provides information about the structural basis of transglycosylation efficiency
83 and product specificity of TmLac. This information will be valuable to obtain new

84 versions of GH2 enzymes capable to synthesize GOS with higher yields or modified
85 chemical structure.

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88 **MATERIALS AND METHODS**

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90 **Strains and culture conditions.**

91

92 *Escherichia coli* XL1-Blue (Stratagene) was the host strain for standard DNA
93 manipulations. Overproduction of TmLac mutant enzymes was carried out in *E.*
94 *coli* XRA. This strain was obtained by transformation of *E. coli* XL1-Blue with the
95 pRARE2 plasmid, which encodes a set of tRNAs which are deficient in *E. coli* and
96 confers resistance to chloramphenicol. pRARE2 was isolated from the *E. coli* strain
97 Rosetta2 (Novagen). Transformants were grown at 37 °C in LB media (0.5% yeast
98 extract, 1% peptone, 0.5% NaCl) supplemented with 100 mg/L ampicilin and, in
99 the case of XRA transformants, 68 mg/L chloramphenicol, with 2% agar for solid
100 media.

101

102 **Cloning and mutagenesis.**

103

104 Site-directed mutagenesis was carried out by PCR, using as template the
105 plasmid TmLac-pQE carrying the gene encoding the wild-type version of TmLac¹⁹,
106 the oligonucleotides showed in Table S1 (supplementary material), and a
107 polymerase with proofreading activity (Phusion DNA polymerase, Thermo
108 Scientific), following the procedure from Hemsley et al²⁰. The resulting mutant

109 genes were sequenced by using ABI Prism BigDye Terminator cycle sequencing kit
110 (Applied Biosystems), and the labeled products were analysed at the Sequencing
111 Service of the University of Valencia (Spain) using a 3730 DNA analyzer (Applied
112 Biosystems). Each plasmid was introduced in *E. coli* XRA strain to optimize gene
113 expression.

114

115 **Expresion of TmLac.**

116

117 *E. coli* transformants were grown in 2XTY (1% yeast extract, 1.5% triptone,
118 0.5% NaCl) supplemented with 100 mg/L ampicillin and 68 mg/L chloramphenicol
119 at 37°C up to an optical density at 600 nm of 0.6 before the induction with 1 mM
120 IPTG (isopropyl β -D-galactopyranoside) at 16 °C during 14 hours. Cultures were
121 centrifuged at 2400 x g for 10 min at 4 °C, and the cells were concentrated 100-fold
122 by resuspending in buffer A (20 mM phosphate, 10 mM imidazole, 500 mM NaCl;
123 pH 7.4) supplemented with Complete EDTA-free protease inhibitor cocktail
124 (Roche). Cells were broken by sonication (5 cycles of 15s at 185 W and 0.5 Hz,
125 followed by 30 s at rest) with a Labsonic from Braun-Biotech keeping the samples
126 on ice. The extract was clarified by centrifugation (15000 g for 20 minutes at 4°C)
127 and kept at 4°C.

128

129 **Enzyme assays.**

130

131 Total β -galactosidase activity was assayed by incubating the clarified
132 extract with 25% (w/v) lactose in reaction buffer (50 mM phosphate pH6.5, 10 mM
133 NaCl, 1 mM $MgCl_2$) at 75°C for 30 min. As previously mentioned, GOS yields

134 increase with higher lactose concentrations, and therefore a lactose concentration
135 close to its limit of solubility at the assay temperature was chosen. The reaction
136 was stopped by heating at 95 °C for 10 min and the amount of released glucose
137 was analysed with a glucose assay kit (Sigma). One unit of activity was defined as
138 the amount of extract that produces 1 µmol of glucose per minute.

139 Analysis of transglycosylating activity was carried out by incubation of the
140 enzyme extracts (0.7-0.8 U/mL) with 25% (w/v) lactose in reaction buffer at 75°C.
141 The reaction was stopped at different times to determine released glucose or GOS
142 production. The pattern of oligosaccharides was analysed by high performance
143 anion exchange chromatography, coupled to a pulsed amperometric detector
144 (Dionex, Thermo Fisher Scientific) equipped with a CarboPac PA-100 column as
145 previously described¹⁹. β-3'-galactosyl-lactose and β-6'-galactosyl-lactose
146 standards were kindly provided by Francisco Javier Cañada.

147

148 **Analysis of enzyme thermal stability.**

149

150 Clarified cell extracts of *E. coli* were subjected to a heat-shock treatment
151 which was carried out by incubating 200 µL of the extract at 85 °C for 5 minutes.
152 Soluble proteins after heat-shock were recovered after centrifugation at 19000 g
153 for 20 minutes and analysed by SDS-PAGE, as previously described¹¹ in parallel to
154 untreated samples.

155

156 **Bioinformatic tools.**

157

158 Multiple sequence analysis was carried out with ClustalW²¹. Molecular
159 modelling was performed with the I-Tasser server²². The Pymol software (Delano
160 Scientific LLC 2006) was used for structural analysis and visualization of modelled
161 structures. Docking analysis between β -3'-galactosyl-lactose and the modelled
162 TmLac was carried out with the program Autodock4²³
163 (<http://autodock.scripps.edu/references>) (see supplementary material for
164 details). The coordinates of β -3'-galactosyl lactose were obtained with GLYCAM
165 (<http://www.glycam.org>).

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167

168 **RESULTS**

169

170 **Structural modelling and mutant design.**

171

172 A model of TmLac on which the design of mutations that could affect
173 transglycosylation would be based upon, was constructed. The structure of most
174 GH2 β -galactosidases is composed by four β -sandwich domains surrounding the
175 central TIM barrel that harbours the catalytic residues. The template with highest
176 sequence identity (ca. 40 %) was the β -galactosidase encoded by the *lacZ* gene of *E.*
177 *coli* (PDB code 3CZJ), hereafter Eclac. Interestingly, TmLac is very similar to Eclac
178 except that it shows an extra C-terminal domain composed of about 100 residues
179 with a predicted β -sandwich fold (Figure 1A). This domain, which is present in β -
180 galactosidases from *Thermotoga* species but not in other homologous enzymes
181 (our own observation), was modeled using as template a CARDB-like domain from
182 *Pyrococcus furiosus*, with a significant (ca. 20%) sequence identity. The C-score

183 value in both cases was higher than -1.5, which is considered the threshold for
184 structural models with correct topology²².

185 Inspection of the active site revealed that residues involved in catalysis or in
186 substrate binding in EcLac are highly conserved (70% identity) in TmLac,
187 rendering a good quality model for this region. In order to identify potential
188 residues involved in transgalactosylation, a docking analysis was carried out using
189 β -3'-galactosyl-lactose as ligand, since this is the major GOS synthesized by wild-
190 type TmLac¹⁹. The complex of TmLac with β -3'-galactosyl-lactose (Figure 1B) may
191 be considered as an analogue of the reaction intermediate where the enzyme is
192 covalently linked to a galactosyl moiety and a new lactose molecule binds to the
193 active site to act as the galactosyl acceptor (Figure S1). According to this model,
194 subsite -1, comprising residues that bind the sugar moiety at the non-reducing
195 end²⁴, would be conformed by D191, H369, N440, E441, E507, H510, W538 and
196 N574. Most of these residues are highly conserved among GH2 β -galactosidases
197 and are associated to essential roles in catalysis²⁵⁻³⁰. Moreover, mutations at many
198 of these positions, including the putative nucleophile and acid/base catalyst (E507
199 and E441, respectively), rendered inactive versions of EcLac²⁵⁻²⁹. Therefore, these
200 residues were discarded as mutagenesis targets. Only N574 showed some
201 sequence divergence among GH2 enzymes, with Asp at the equivalent position in
202 some cases (Figure 1C). The possible functionality of this substitution, either for
203 transglycosylation efficiency or product specificity was investigated with mutant
204 N574D. Furthermore, mutants N574A and N574S were generated. The rationale
205 behind this was that the disruption of a hydrogen bond with the galactosyl moiety
206 covalently linked to the enzyme in the reaction intermediate may increase the
207 rotational freedom of this group to be coupled to the acceptor lactose. Subsites +1

208 and +2, binding the galactosyl and glucosyl moiety of the acceptor lactose,
209 respectively, would involve fewer residues than subsite -1 and should not be as
210 critical for β -galactosidase activity. W959 and N95 are in contact to both the
211 galactosyl and glucosyl groups of the acceptor lactose, whereas F571 and D568
212 may conform subsite +2. Interestingly, W959 is conserved in bicistronic β -
213 galactosidases composed by two different polypeptides, where this residue is
214 located in subunit LacM, different from LacL, which contains the catalytic residues
215 (Figure 1C). In some monocistronic β -galactosidases, like that from *Kluyveromyces*
216 *lactis*, the equivalent position is occupied by a cysteine (Figure 1C). The
217 functionality of W959, F571 and D568 in the transgalactosylation reaction was
218 tested with mutants W959C, W959F, W959A, F571L and D568A. Finally, V93 and
219 V94 are located close (around 4 and 7 Å, respectively) to the terminal glucosyl
220 moiety (Figure 1B). These residues are substituted by Thr or Gln in other β -
221 galactosidases with high transgalactosylating activity (Figure 1C). These more
222 extended side chains may establish additional hydrogen bonds to the acceptor
223 lactose or facilitate the binding of a trisaccharide as alternative acceptor to
224 generate a tetrasaccharide. Mutants V93T, V94T and V94N were generated to
225 explore this hypothesis.

226

227 **Analysis of heterologous gene expression and thermal stability of mutant** 228 **enzymes.**

229

230 Extracts of *E. coli* transformants expressing different TmLac mutants were
231 analysed by SDS-PAGE to evaluate the production of enzyme. Thermal stability of
232 the mutant versions was assessed by a parallel analysis of the remaining soluble

233 protein after heat treatment. All TmLac mutants had a expression efficiency and
234 thermal resistance similar to the wild-type, with a few exceptions (supplementary
235 Figure S2). Mutants N574S and W959F were produced in significantly lower
236 amounts than the wild-type enzyme. Despite of this, the percentage of enzyme
237 recovered after heat treatment was similar to that obtained with the wild-type.
238 Therefore, none of the introduced mutations seem to cause drastic structural
239 changes altering the overall stability of the enzyme.

240

241 **Activity of transformants expressing mutant TmLac versions.**

242

243 Glucose release by TmLac activity is concomitant to both hydrolysis and
244 transglycosylation (Figure S1). Therefore, initial velocity of glucose production can
245 be used as an estimation of the overall activity of the enzyme. Substitution of Asn
246 574 by Ala or Ser caused a significant decrease in the global activity of the enzyme
247 per unit of total protein in the extract (to 31 % and 8 % of the wild-type,
248 respectively), whereas replacement by Asp did not have a significant effect (Table
249 1). The drastic reduction of activity in the N574S mutant seems to be correlated
250 with the decreased synthesis of enzyme observed in Figure S2. The three
251 mutations tested at W959 were associated with a decrease in the overall specific
252 activity of the enzyme (Table 1), although the decrease of W959F activity may be
253 simply the result of the lower expression of this mutant enzyme. On the contrary,
254 substitution of W959 by non-aromatic residues (Cys or Ala) caused a significant
255 decrease in activity (to 21% and 45% of the wild-type, respectively) despite
256 showing a similar enzyme amount. Activity of the rest of mutants was similar to
257 that of the wild-type. Therefore, comparing relative activity values (Table 1) with

258 relative enzyme amounts (Figure S2), we conclude that only substitutions N574A,
259 W959C and W959A cause a significant detriment in TmLac activity per unit of
260 enzyme mass.

261

262 **Analysis of transgalactosylation efficiency and GOS profile of mutant**
263 **enzymes.**

264

265 The kinetics of β -3'-galactosyl-lactose and β -6'-galactosyl-lactose synthesis
266 was evaluated for the wild-type enzyme (supplementary Figure S3A). In parallel,
267 glucose release was determined as an estimation of enzyme activity, revealing that
268 TmLac is progressively inactivated (Figure S3B). After 3 hours of reaction, the rate
269 of glucose release was around 20 % of the initial velocity. Since neither substrate
270 availability nor enzyme stability is compromised after this incubation time¹⁹, this
271 may be a consequence of enzyme inhibition by product accumulation, as reported
272 for other β -galactosidases^{31,32}. Consequently, GOS synthesis was also slowed down
273 as reaction progressed, with a much slower rate after 3 hours of reaction (Figure
274 S3A). As previously reported¹⁹, β -3'-galactosyl-lactose was preferentially
275 synthesized over β -6'-galactosyl-lactose. In order to compare GOS yields from
276 different enzymatic variants, the same units of enzyme (i.e. initial glucose release
277 per unit of time) were used. GOS production was determined after 5 hours of
278 reaction and the kinetics of glucose release was monitored in parallel as a control.
279 All the enzymatic versions synthesized the same GOS types as the wild-type
280 enzyme (β -3'-galactosyl-lactose and β -6'-galactosyl-lactose). GOS with higher
281 degree of polymerization were not detected in any case.

282 Substitutions N574S and N574A, but not N574D yielded an increase in the
283 synthesis of β -3'-galactosyl-lactose, compared to the wild-type enzyme (around
284 30% and 40% higher, respectively), whereas β -6'-galactosyl-lactose synthesis was
285 not significantly affected (Figure 2A). Similarly, the mutant F571L also showed an
286 increased β -3'-galactosyl-lactose synthesis (around 40%) (Figure 2B).

287 Mutations at Asp 568, Val 93 and Val 94 did not affect the
288 transgalactosylation efficiency or product specificity of the enzyme (Figure 3A and
289 3C). In contrast, substitutions W959C and W959A caused a drastic reduction
290 (around 80%) in β -3'-galactosyl-lactose synthesis (Figure 3B). This effect was not
291 so remarkable in β -6'-galactosyl-lactose synthesis. Therefore, β -6'-galactosyl-
292 lactose to β -3'-galactosyl-lactose ratio was reduced from 2.6, obtained with the
293 wild-type enzyme, to 1 and 0.7, for W959C and W959A, respectively. The W959F
294 mutation did not cause a significant change in the transgalactosylating properties
295 of the enzyme.

296 Substitutions with higher increase in transgalactosylating efficiency were
297 combined generating mutants F571L/N574S and F571L/N574A. The resulting
298 enzymes showed a significant reduction in global activity (Table 1), despite their
299 expression was similar to that of the wild-type (Figure S2). The synthesis of β -3'-
300 galactosyl-lactose by F571L/N574S and F571L/N574A was increased around 90%
301 and 70% compared to the wild-type enzyme, respectively (Figure 2C). This
302 represented a significant increment compared to the single mutants F571L and
303 N574S.

304

305

306 **DISCUSSION**

307

308 GOS synthesis has become an active research topic due to the increasing use
309 of these compounds in functional food and nutraceuticals. GOS with $\beta(1,3)$, $\beta(1,4)$
310 or $\beta(1,6)$ linkages can be obtained with enzymes from different microbial sources⁵.
311 Production of commercial GOS is carried out with enzymes from *Bifidobacterium*
312 *bifidum* for $\beta(1,3)$ -GOS, *Cryptococcus laurentii* or *Bacillus circulans* for $\beta(1,4)$ -GOS
313 and a mixture of *Aspegillus oryzae* and *Streptococcus thermophilus* for $\beta(1,6)$ -GOS³³⁻
314 ³⁹. Production of GOS in high yield and with a broad chemical repertoire, are two
315 major biotechnological challenges. A few cases of enzyme engineering to increase
316 GOS yield, using GH1 and GH42 enzymes, have been reported⁴⁰⁻⁴². So far GH2
317 enzymes have not been manipulated with this purpose. Therefore, the aim of this
318 study was to find out the role that specific residues in the active site of a GH2
319 glycosidase might have in the transglycosylating activity and specificity of the
320 enzyme. For this purpose we chose a thermoresistant β -galactosidase from the
321 bacterium *Thermotoga maritima*. We undertook rational design of mutations
322 based on the analysis of homologous sequences of GH2 enzymes with
323 transglycosylating activity, and docking analysis of a structural model of TmLac
324 with the transglycosylation product β -3'-galactosyl-lactose.

325 Aminoacid substitutions within the -1 subsite may disrupt a hydrogen bond
326 with the galactosyl moiety in the covalent complex formed in the reaction
327 intermediate. Accordingly, mutations N574S and N574A, caused a significant
328 decrease in overall activity (Table 1), but in contrast transgalactosylation activity
329 was increased with a substantial increment (30-40%) in the synthesis of β -3'-
330 galactosyl-lactose. In the mutants, the loss of a stabilising interaction would
331 decrease the affinity of the enzyme by the substrate and the stability of the

332 covalent intermediate, explaining the decrease in activity. However, a higher
333 rotational freedom of the galactosyl moiety in the covalent complex could favour
334 its transfer to an acceptor lactose molecule (Figure 4A). Our results also suggest
335 that this higher flexibility would specifically facilitate coupling to the acceptor
336 lactose through a $\beta(1,3)$ linkage (Figure 2A). Mutant N574D, which involves the
337 substitution of a carboxy group by an amide with the same side chain length, may
338 still be able to keep such polar contact with the galactosyl moiety. In agreement
339 with this, no changes were found in the protein expression (Figure S2), activity
340 (Table 1) or transglycosylating efficiency (Figure 2A) of this mutant.

341 Docking analysis suggests that residues W959 and F571 may conform an
342 aromatic residue platform for binding the acceptor lactose (Figure 1B). In
343 agreement with this, substitutions of W959 by non-aromatic residues had a drastic
344 effect in transgalactosylation. The homologous residue in EcLac (W999) has been
345 assigned a double role in the catalysis of the enzyme, for lactose binding in the so-
346 called “shallow” mode, and for glucose binding in the galactosyl covalent
347 intermediate for allolactose synthesis⁴³⁻⁴⁵. Our results suggest that this residue is
348 also involved in binding the lactose molecule that acts as an acceptor in the
349 transgalactosylating reaction. The effect of W959 substitutions was specially
350 remarkable for β -3'-galactosyl-lactose synthesis (Figure 3B). The $\beta(1,3)$ to $\beta(1,6)$
351 synthesis specificity decreased from 2.6 in the wild-type enzyme to 1 and 0.7 in
352 W959C and W959A, respectively. Interestingly, β -galactosidase from *K. lactis*, with
353 a Cys residue in the homologous position synthesizes preferentially β -6'-
354 galactosyl-lactose⁴⁶, suggesting that this residue may be critical for the specific
355 synthesis of β -3'-galactosyl-lactose.

356 Substitution of highly conserved F571 by a non-aromatic residue (F571L),
357 resulted in a significant increase in the synthesis of β -3'-galactosyl-lactose (Figure
358 2B). Modelling studies suggest that substitution F571L may allow the rotation of
359 W959, which seems to be sterically impeded in the wild-type enzyme (Figure 4B).
360 Higher flexibility may facilitate a better orientation of W959 as a platform for
361 binding the acceptor lactose molecule, favouring the synthesis of a β (1,3) linkage
362 (Figure 2B). According to our assumptions, double mutation at F571 and N574
363 (F571L/N574S or F571L/N574A) would simultaneously confer higher flexibility of
364 the galactosyl group at the covalent intermediate (by disrupting the putative
365 hydrogen bond with N574) and a better docking of the acceptor lactose by
366 reorienting W959, resulting in a more favourable coupling of both molecules. This
367 double mutation has increased the transgalactosylating efficiency of the wild-type
368 enzyme up to two-fold.

369 GOS yields obtained with β -galactosidases range between 20-200 g/L, with
370 different chemical profiles⁵. Among thermoresistant β -galactosidases, GH1
371 enzymes from *S. solfataricus* and *P. furiosus* synthesize around 40-50 g/L of
372 trisaccharides consisting of β -3'-galactosyl-lactose and β -6'-galactosyl-lactose in a
373 2:1 ratio¹⁵ whereas GH2 β -galactosidase from *S. thermophilus* produced galactose
374 disaccharides but no trisaccharides¹⁴. Site-directed mutagenesis of the GH2 TmLac
375 enzyme reported in this communication increased GOS yield in about 50% (from
376 30 g/L to 45 g/L) with a final β -3'-galactosyl-lactose to β -6'-galactosyl-lactose
377 ratio of 4:1. Due to the high degree of conservation of F571 and N574 among the
378 GH2 β -galactosidases, our results could be extrapolated to other enzymes,
379 including those with different product specificity. Equivalent mutations may be
380 attempted to increase the yield of different GOS products.

381

382

383 **ACKNOWLEDGMENT**

384

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386 galactosyl-lactose, used as standards.

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388

389 **SUPPORTING INFORMATION**

390

391 Methodological details for docking analysis; Table S1, Oligonucleotides used as
392 primers for site-directed mutagenesis; Figure S1, Reaction scheme of hydrolysis
393 and transgalactosylation; Figure S2, Analysis of expression of TmLac mutants in *E.*
394 *coli* by SDS-PAGE; Figure S3, Kinetics of glucose release and GOS synthesis by wild-
395 type TmLac. This material is available free of charge via the Internet at
396 <http://pubs.acs.org>

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554

555 **FIGURE CAPTIONS**

556

557 **Figure 1.** Structural analysis of TmLac and design of mutations. **A.** A schematic
 558 representation of the domain arrangement of TmLac is depicted on top, showing
 559 the central α/β barrel (α/β) surrounded by 5 β -sandwich domains ($\beta 1$ to $\beta 5$). Left
 560 panel: Model of the main body of TmLac (residues 1-983) (C-score: -1.11). Right
 561 panel: Model of the C-terminal domain (residues 984-1084) (C-score: -1.30). **B.**
 562 Structural detail of the active site highlighting the catalytic residues (red) and the
 563 residues targeted for site-directed mutagenesis (orange). β -3'-galactosyl-lactose is
 564 depicted with the galactosyl moiety at the non-reducing end in blue. The rest of the
 565 molecule, coloured in violet, represents the lactosyl moiety, acting as acceptor of
 566 the galactosyl group during transgalactosylation. Dashed lines indicate distances of
 567 3-4 Å between non-aromatic residues subjected to mutagenesis and the modeled
 568 ligand. **C.** Sequence alignment of TmLac with other GH2 β -galactosidases with
 569 transgalactosylating activity. The sequence stretches around the residues targeted
 570 for site-directed mutagenesis are shown. The relative position of these stretches
 571 within their corresponding domain is indicated on top. Genebank codes for the
 572 indicated proteins are: *T. maritima*: AAD36268.1, *E. coli*: AAA24053.1, *L.*
 573 *delbrueckii*: CAI98003.1, *K. lactis*: AAA35265.1, *B.*
 574 *longum_sub_infantis*:_AAL02052.1, *L. acidophilus* LacL:_ABK59934.1, *L.*
 575 *acidophilus*_LacM:_ABK59935.1, *L. reuteri*_LacL:_ ABF72116.1, *L.*
 576 *reuterii*_LacM:_ABF72117.2, *L. plantarum*_LacL:_CAZ66936.1, *L.*
 577 *plantarum*_LacM:_CAD65570.1

578

579 **Figure 2.** Effect of substitution of residues F571 and N574 on the synthesis of
580 galacto-oligosaccharides (right panels) β -3'-galactosyl-lactose (black bars) and β -
581 6'-galactosyl-lactose (grey bars). Error bars represent standard deviation of
582 triplicates. Asterisks indicate significant differences ($p < 0.01$) compared to wild-
583 type data. Kinetics of glucose release is shown on the left panels as a control.

584

585 **Figure 3.** Effect of substitution of residues D568, W959, V93 and V94 on the
586 synthesis of galacto-oligosaccharides (right panels) β -3'-galactosyl-lactose (black
587 bars) and β -6'-galactosyl-lactose (grey bars). Error bars represent standard
588 deviation of triplicates. Asterisks indicate significant differences ($p < 0.01$)
589 compared to wild-type data. Kinetics of glucose release is shown on the left panels
590 as a control.

591

592 **Figure 4.** Structural model of mutants with higher transgalactosylating efficiency.
593 β -3'-Galactosyl-lactose is depicted with the galactosyl moiety at the non-reducing
594 end in light brown, and the lactosyl group acting as acceptor coloured in violet. **A.**
595 Residues interacting with the galactosyl moiety at the non-reducing end are
596 highlighted in green. The residues substituting N574 in the N574D and N574S
597 mutants are overlapped and depicted in blue and orange, respectively. Dashed
598 lines indicate putative hydrogen bonds at this specific position. **B.** The position of
599 W959 and F571 in the wild-type enzyme is shown in green. The structure F571L
600 mutant (orange) is overlapped and shows that the side chain of W959 is not
601 sterically impeded to rotate to a different position.

Table 1

Enzyme	Total activity [$\mu\text{mol Glc} \cdot \text{min}^{-1} \cdot \mu\text{g protein}^{-1}$]
	(% of wild type activity)
wt	4.4 \pm 0.9 (100)
V93T	4.15 \pm 0.08 (94)
V94T	3.8 \pm 0.3 (86)
V94Q	4.3 \pm 0.7 (98)
D568S	4.8 \pm 0.2 (110)
D568A	5.17 \pm 0.17 (120)
F571L	3.1 \pm 0.2 (70)
N574A	1.4 \pm 0.3 * (31)
N574S	0.36 \pm 0.06 * (8)
N574D	4.3 \pm 0.3 (97)
W959F	0.22 \pm 0.06 * (5)
W959C	0.94 \pm 0.05 * (21)
W959A	2.0 \pm 0.2 * (45)
F571L/N574S	0.41 \pm 0.03 * (9)
F571L/N574A	0.30 \pm 0.02 * (7)

* statistical difference ($p < 0.01$) with wild-type value.

Figure 1

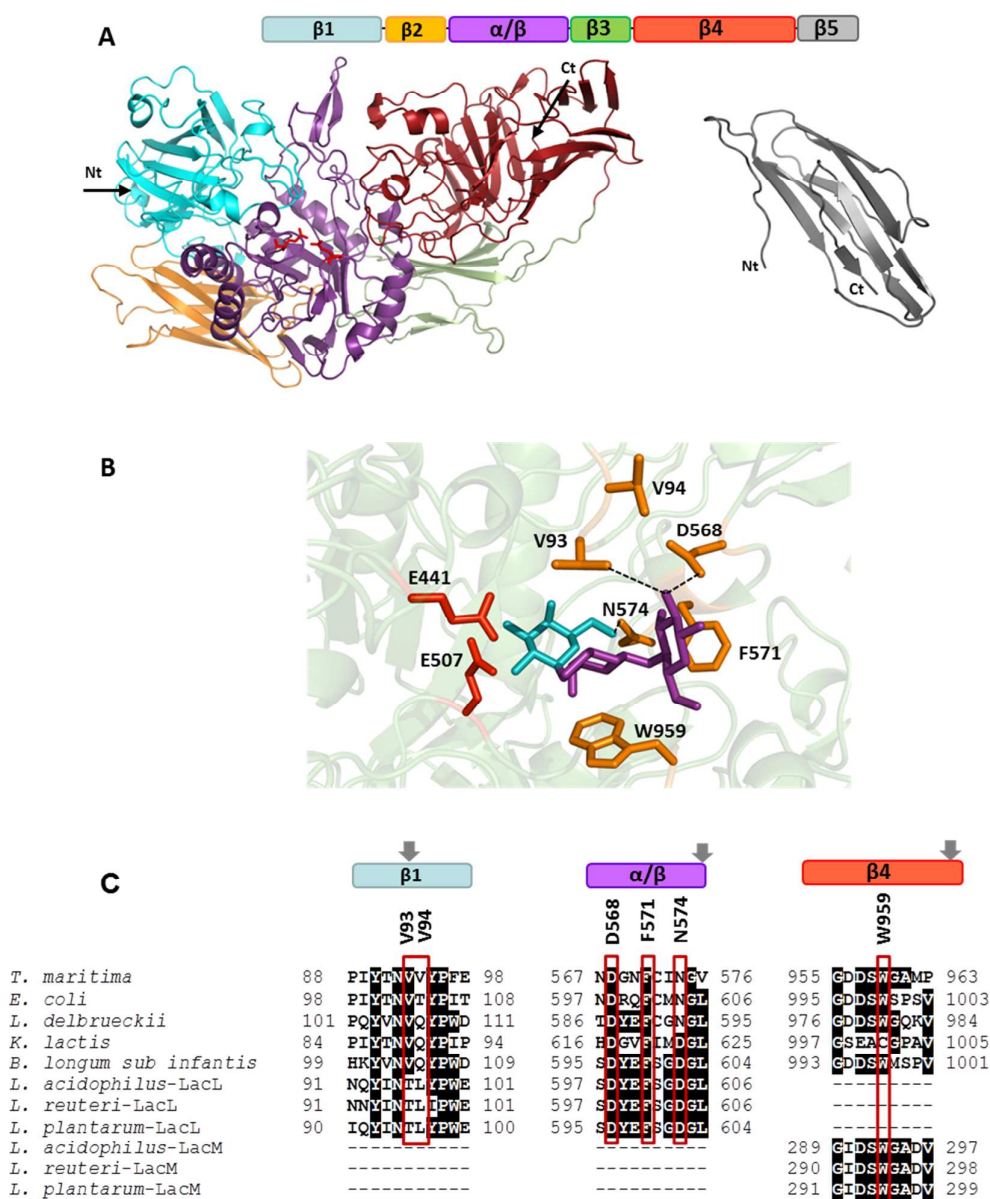


Figure 2

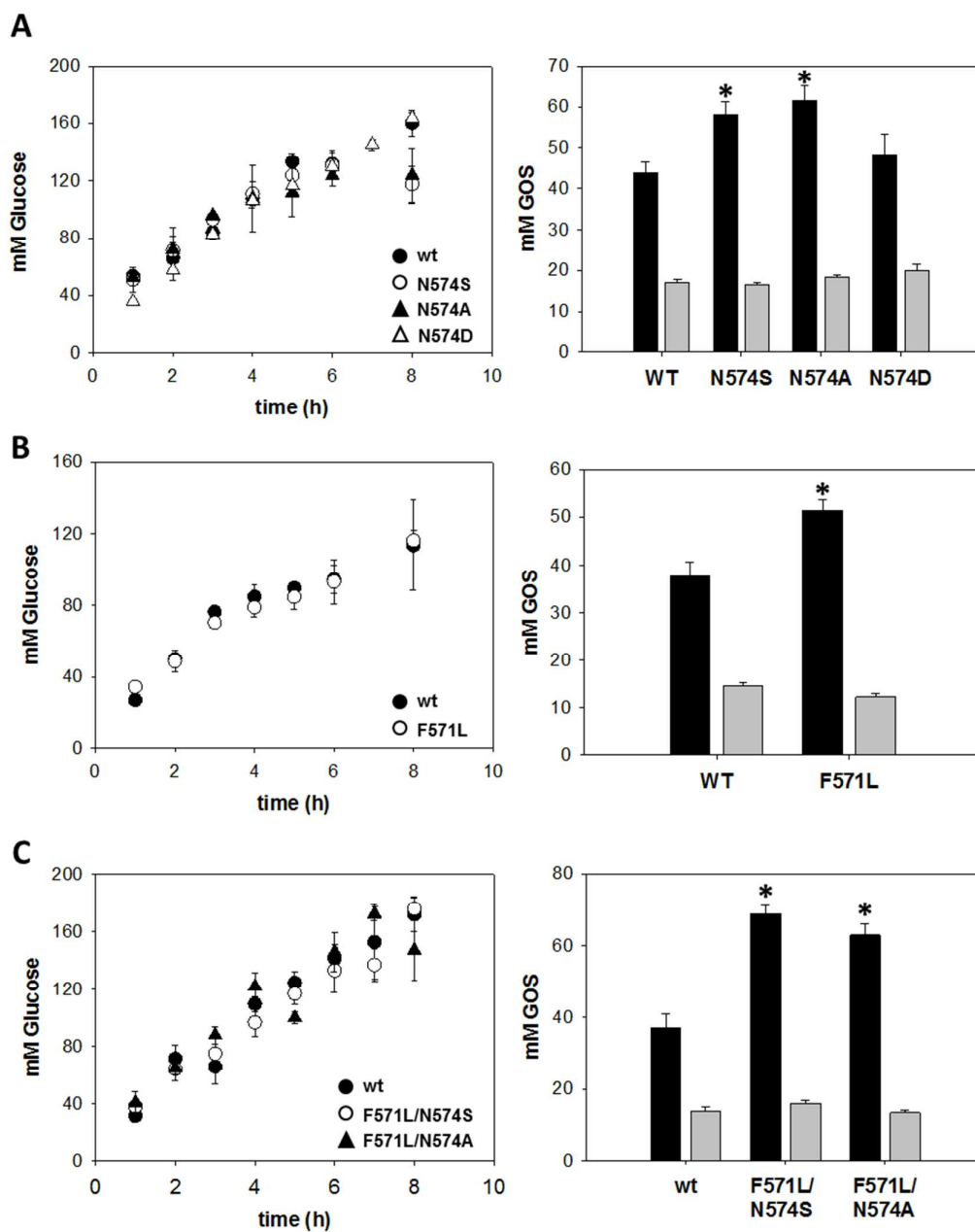


Figure 3

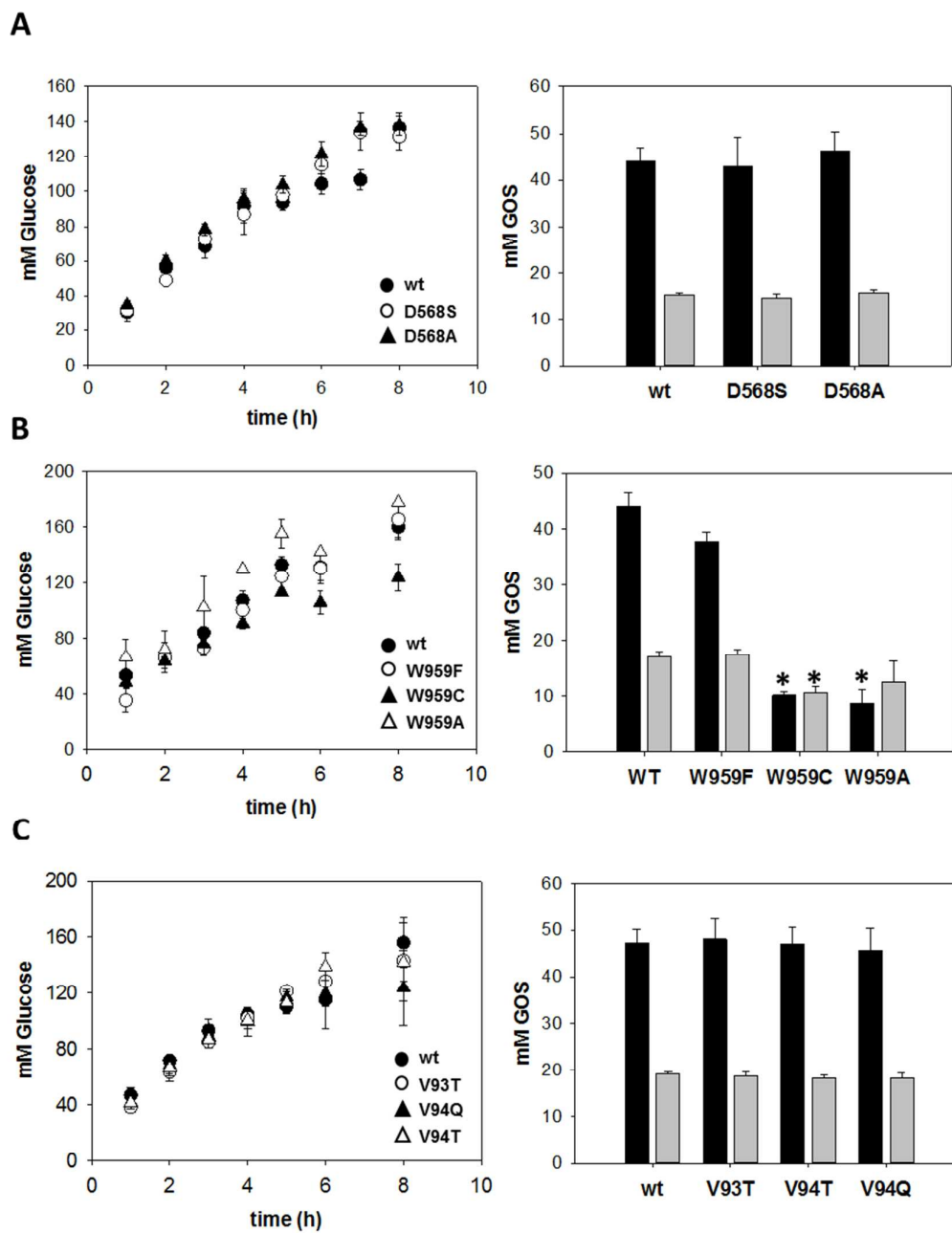
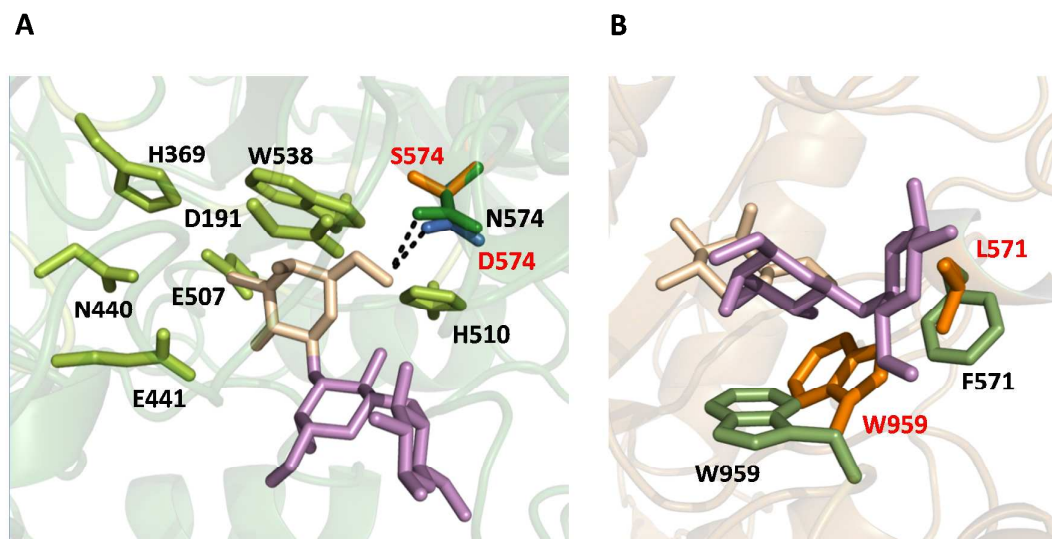


Figure 4



GRAPHIC FOR TABLE OF CONTENTS

