The binding mechanism of *Streptococcus suis* accessory virulence factor and adhesin SadP to globotetraosylceramide

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Abstract

Streptococcus suis is part of the pig commensal microbiome and a major pig pathogen causing pneumonia and meningitis, occasionally also in humans. The genomic analysis divides *S. suis* to asymptomatic

carriage, respiratory and invasive systemic strains with distinct genomic signatures. The virulence factor *S. suis* adhesin P (SadP) recognizes the galabiose Galα1–4Gal-oligosaccharide. Based on the oligosaccharide fine specificity, SadP can be divided into subtypes P_N and P_O. We show that subtype P_N is distributed in the systemic strains. Both types of SadP are shown to predominantly bind to pig lung globotriaosylceramide (Gb3). SadP adhesin from virulent subtype P_N strain also binds to globotetraosylceramide (Gb4). Mutants of type P_N SadP adhesin were constructed into the galabiose-binding domain and analyzed to reveal the mechanism for Gb4 binding. Functional and structural analysis of type P_N SadP mutants showed that amino acid N285 of the galabiose-binding site was required for binding to Gb4 and strikingly was also required for interaction with the glycomimetic inhibitor phenylurea-galabiose. Molecular dynamics simulations provided further insight into the role of N285 for Gb4 and phenylurea-galabiose binding, suggesting additional hydrogen bonding to terminal GalNAc of Gb4 and urea-group. Importantly, type P_N SadP adhesin expressed by strains causing invasive systemic disease binds via a distinct amino acid N285 mediated mechanism to Gb4. Thus, this molecular mechanism of type P_N SadP binding is a candidate for selectively targeting *S. suis* meningitis without interfering with commensal strains, which opens up new venues for developing intervention strategies against this pathogen.

Author summary

Streptococcus suis is a major pig pathogen, which occasionally also causes meningitis in humans. Despite the wealth of existing genomic data, the mucosal surface colonizing mechanisms of these streptococci are poorly understood. *S. suis* population evolves rapidly and is prone to acquire antibiotic resistance factors and could hamper the efficacy of vaccines by serotype replacement. The accessory genomes of specific subpopulations are expected to encode factors that are associated with particular ecological niches and enhanced pathogenicity. By identification of such accessory factors we could aim to develop clinical interventions targeting the pathovariants while leaving commensal bacteria unharmed, which would mitigate harmful serotype replacement. In this study we identified by both genomic and structure-function analysis the accessory *S. suis* virulence factor SadP as specific for population causing meningitis. The presented data

provide an insight into a molecular mechanism how a single amino acid change can cause a functional divergence in host cell glycolipid receptor binding in emerged meningitis pathovariant of streptococci. The results can be utilized to design population- and ecology aware means to target infectious disease caused by this pathogen.

Introduction

Bacterial adhesion to host cell surfaces is a prerequisite for infectious disease. Host cell surfaces are heavily covered by surface carbohydrates that form the glycocalyx layer [1,2]. Pathogenic bacteria and their toxins can treacherously exploit carbohydrates to attach and invade cells. One example is a group of endogenous glycosphingolipids, known as globo series membrane glycosphingolipids, which are grouped based on the Galα1–4Gal (galabiose) structure. Globo series glycolipids are cellular receptors for toxic action of Verotoxins and for the attachment of bacteria, such as uropathogenic *Escherichia coli*, *Streptococcus suis* and *Pseudomonas aeruginosa* [3–6].

Since antibiotics are increasingly losing their power, alternative antimicrobial compounds need to be invented and developed [7,8]. Transformation of bacteria to antibiotic-resistant forms can be overcome by developing compounds that block *in vivo* colonization and virulence factors without killing the pathogens. Cell surface carbohydrate glycomimetics are the most tempting pipeline for the development of new generation of antimicrobials [9,10].

Streptococcus suis is an important pig pathogen causing septicemia, pneumonia and meningitis. It also causes severe zoonotic meningitis. Recent data show that it is a major pathogen of pigs and produces an alarmingly vast array of factors causing high antibiotic resistance [11]. The pathogenic types of *S. suis* are efficiently transmitted from host to host and the bacterium can be frequently isolated from the tonsils as a carrier strain. However, unfortunately predisposed by environmental factors such as overcrowding and synergistic activities with other keystone pathogens [12], *S. suis* invades the host tissues and by expressing both soluble and cell wall bound virulence factors aggravates tissue damage in the critical organs and causes death [13,14].

95 *S. suis* strains expressing SadP adhesin bind specifically to Galα1–4Gal-containing oligosaccharides
96 [15]. Interestingly, galabiose-binding strains can be divided to subtypes P_N and P_O based on how they recognize
97 galabiose-containing carbohydrates in hemagglutination assays [16]. Type P_N strains recognize galactose, *N*-
98 Acetylgalactosamine, and the ceramide-linked oligosaccharides Gb3 (Galα1–4Galβ1–4Glc) and Gb4
99 (GalNAcβ1–3Galα1–4Galβ1–4Glc). Subtype P_O recognizes only galactose and preferentially binds to Gb3.
100 Recently, *S. suis* galabiose-binding adhesin SadP was identified as an LPNTG-anchored cell-wall protein [6].
101 The carbohydrate interaction of SadP has been shown to mediate *S. suis* binding to pharyngeal epithelium and
102 intestinal epithelial cells [16,17]. In an *S. suis* murine infection model, the mice deficient in globotriaosyl
103 ceramide (Gb3) expression (knockout in alpha-1,4-galactosyltransferase A4GALT) developed less severe
104 brain inflammation and injury [18]. Recently, low picomolar concentrations of glycomimetic 3-phenylurea-
105 galabiose-containing dendrimers were shown to block *S. suis* SadP (type P_N) adhesin binding activity [19].
106 However, the molecular basis of differences in galabiose-binding mechanisms of SadP adhesins have remained
107 elusive. In the present study, we have characterized the pig Galα1–4Gal-containing receptors in porcine lung
108 tissue using mass spectrometry and thin layer chromatography (TLC) overlay assay. The binding mechanism
109 of type P_N, P_O and site-specific mutants of type P_N SadP to globosyl oligosaccharides were studied. The results
110 show that *S. suis* SadP specifically recognizes globo series glycolipids isolated from the pig lungs and that
111 specific amino acid changes in the proximity of the galabiose-binding site have an effect on the SadP type P_N
112 and P_O specificity.
113

114 # Results

115 ## Sequence analysis of SadP subtypes P_N and P_O, structural modelling and distribution in *S.*
116 ## *suis* systemic, respiratory and non-clinical strains

117 SadP adhesins are 80 kDa LPNTG-anchored cell-wall proteins, which contain the signal sequence for
118 secretion, the N-terminal galabiose binding domain, tandem repeat domains and C-terminal LPNTG cell-wall
119 anchor domain [6]. The galabiose-binding domains have no significant sequence homologs to other bacterial
120 proteins or to other carbohydrate binding domains. The SadP N-terminal galabiose-binding domains (aa125-

121 328) of hemagglutinating type P_N and type P_O strains were cloned, sequenced and compared with multiple
122 alignment (Fig 1A). The structure consists of three α-helixes and ten β-strands (β1-β10) that form a β-
123 sandwich core domain. The sequences of the N-terminal galabiose-binding domain of type P_N SadP adhesins
124 are 100 % identical and are found in virulent serotype 2 strains. There is more sequence variation in the
125 galabiose-binding site of type P_O SadP. The carbohydrate binding sites of types P_N and P_O are highly conserved
126 (Fig 1B). Interestingly there is a conservative change at position 285 from asparagine to aspartate, which could
127 have an effect for the interaction of type P_N SadP with GalNAcβ1–3Galα1–4Gal-oligosaccharide.

128 The distribution of *sadP* genes in clinical and non-clinical strains analyzed with core genome phylogeny
129 analysis is shown in Figure 1C. The gene of *sadP* type P_N was found in 91.9 % of systemic *S. suis* isolates
130 (88.1 % of strains isolated from brain), whereas only less than 1 % of strains had the gene encoding *sadP* type
131 P_O. Based on the maximum-likelihood core genome phylogeny analysis the type P_N SadP gene is found in
132 clonal virulent *S. suis* strains, which mostly belong to serotype 2. Interestingly, also serotype 14 strains isolated
133 from human meningitis cases have type P_N gene. Type P_O *sadP* was found more frequently in respiratory and
134 non-clinical strains.

135

136 **Purification and analysis of glycosphingolipid composition of porcine lung**

137 The interaction of recombinant SadP with globo series glycolipids has not been thoroughly
138 characterized. Moreover, whether SadP binds to Gb3 or Gb4 isolated from the pig tissues has not yet been
139 directly shown. The glycolipids were isolated from porcine lung and erythrocytes, in order to control that the
140 glycolipids isolated from the lungs were not contaminants derived from the erythrocytes remaining in the
141 lungs. The total non-acid glycosphingolipid fractions were hydrolyzed with endoglycoceramidase II of
142 *Rhodococcus* sp., and the oligosaccharides thereby obtained were analyzed by LC-ESI/MS using graphitized
143 carbon columns. This method gives resolution of isomeric oligosaccharides, and the MS^2 analyses gives
144 complete sequence information and allows differentiation of linkage positions by diagnostic cross-ring $^{0,2}A$-
145 type fragment ions [20].

146 The base peak chromatograms from LC-ESI/MS of the oligosaccharides obtained by hydrolysis of the
147 total non-acid glycosphingolipid fractions of porcine erythrocytes and lung are shown in Figure 2A. By

148 comparison of the retention times and MS^2 spectra of oligosaccharides obtained from reference
149 glycosphingolipids, the major oligosaccharides obtained from porcine lung were tentatively identified as
150 globotriaosylceramide (Gb3, detected as a $[M-H^+]^-$ ion at *m/z* 503) and the blood group H type 2
151 pentasaccharide (H5-2, detected as a $[M-H^+]^-$ ion at *m/z* 852) (Figure 2A). The major oligosaccharides from
152 porcine erythrocytes were globotriaosylceramide (Gb3, detected as a $[M-H^+]^-$ ion at *m/z* 503),
153 globotetraosylceramide (Gb4, detected as a $[M-H^+]^-$ ion at *m/z* 706), and the blood group A type 2
154 hexasaccharide (A6-2, detected as a $[M-H^+]^-$ ion at *m/z* 1055) (Fig 2B). Thus, globotriaosylceramide was found
155 in both samples, but otherwise the two samples were significantly different. The total non-acid
156 glycosphingolipid fraction from porcine lung was thereafter separated by Iatrobeads chromatography. Aliquots
157 of the fractions obtained were analyzed by thin-layer chromatography, and fractions that were coloured green
158 by anisaldehyde were tested for binding of SadP using the chromatogram binding assay. The fractions were
159 thereafter pooled according to the mobility on thin-layer chromatograms and their SadP binding activity.
160

161 **The binding of SadP to neutral glycolipids isolated from porcine lung using TLC overlay**
162 **assay**

163 The separations gave two SadP binding fractions migrating in the triglycosylceramide region. One
164 fraction (0.2 mg) migrating as a single band was denoted fraction PL-1 (Fig 2B, lane 1), and one fraction (0.3
165 mg) migrating as a double band was denoted fraction PL-2 (Fig 2B, lane 2). In addition, one SadP binding
166 fraction (0.4 mg) with compounds migrating as tetraglycosylceramides was obtained (denoted fraction PL-4;
167 Fig 2B, lane 4).
168

169 **LC-ESI /MS of native SadP binding glycosphingolipids from porcine lung**

170 ESI/MS of the native fraction PL-1 gave a major $[M-H^+]^-$ ion at *m/z* 1132 (S1A Fig), indicating a
171 glycosphingolipid with three Hex, and sphingosine with non-hydroxy 24:1 fatty acid. MS^2 of the $[M-H^+]^-$ ion
172 at *m/z* 1132 gave a series of Y ions (Y_0 at *m/z* 646, Y_1 at *m/z* 808, and Y_2 at *m/z* 970) demonstrating a Hex-
173 Hex-Hex sequence combined with sphingosine with non-hydroxy 24:1 fatty acid (S1B Fig).

174 A $[M-H^+]^-$ ion at *m/z* 1132 was also obtained by ESI/MS of the native fraction PL-2 (S2 Fig). Here, the
175 major $[M-H^+]^-$ ion was seen at *m/z* 1022 (S2AB Fig), demonstrating a glycosphingolipid with three Hex, and
176 sphingosine with non-hydroxy 16:0 fatty acid. Also, here a Hex-Hex-Hex sequence was demonstrated by the
177 series of Y ions (Y_0 at *m/z* 536, Y_1 at *m/z* 698, and Y_2 at *m/z* 860) from MS^2 (S2D Fig).
178

179 **Endoglycoceramidase digestion and LC-ESI /MS of SadP binding glycosphingolipids from**
180 **porcine lung**

181 The base peak chromatograms from LC-ESI/MS of the oligosaccharides obtained by
182 endoglycoceramidase digestion of fractions PL-1 and PL-2 were very similar (S3AB Fig). Both had a $[M-H^+]^-$
183 ion at *m/z* 503 which eluted at the same retention time (17.4-18.4 min) as the saccharide obtained from
184 reference globotriaosylceramide (S3C Fig), while the saccharide from reference isoglobotriaosylceramide
185 eluted at 20.0-20.4 min (Figure S3D).
186 MS^2 of the ion at *m/z* 503 of fractions PL-1 and PL-2 gave in both cases two C-type fragment ions (C_1
187 at *m/z* 179 and C_2 at *m/z* 341) identifying a Hex-Hex-Hex sequence (Figure S3EF). A 4-substitution of the
188 internal Hex was demonstrated by the $^{0,2}A_2$ fragment ion at *m/z* 281 [20–22] . Taken together with the similarity
189 to the MS^2 spectrum of reference globotriaosyl saccharide (S3G Fig), this allowed identification of the
190 saccharides of fractions PL-1 and PL-2 as globotriaosyl saccharides (Galα1–4Galβ4Glc).
191 LC-ESI/MS of the oligosaccharides obtained by hydrolysis of fraction PL-4 with *Rhodococcus*
192 endoglycoceramidase II allowed a tentative identification of a globotetraosyl saccharide (GalNAcβ1–3Galα1–
193 4Galβ1–4Glc). This conclusion was based on the following spectral features: First, the base peak
194 chromatogram of fraction PL-4 had a $[M-H^+]^-$ ion at *m/z* 706 (Figure S4A), and MS^2 of this ion (S4B Fig) gave
195 a C-type fragment ion series (C_1 at *m/z* 220, C_2 at *m/z* 382, and C_3 at *m/z* 544), demonstrating a HexNAc-Hex-
196 Hex-Hex sequence. The $^{0,2}A_3$ fragment ion at *m/z* 484 demonstrated a 4-substituted Hex, while the $^{0,2}A_4$ ion at
197 *m/z* 646, and the $^{0,2}A_4$-H_2O ion at *m/z* 628, were derived from cross-ring cleavage of the 4-substituted Glc of
198 the lactose unit at the reducing end. The features of this MS^2 spectrum were very similar to the MS^2 spectrum
199 of the reference globotetraosyl saccharide (S4C Fig)

200 The base peak chromatogram of fraction PL-4 (S4A Fig) also had two $[M-H^+]^-$ ions at *m/z* 998, eluting
201 at 19.4 min and 21.4 min. In both cases MS^2 demonstrated a Fuc.Hex-(Fuc-)HexNAc-Hex-Hex sequence, and
202 the diagnostic ion at *m/z* 348 (S4D Fig) identified a Le^b hexasaccharide, whereas the diagnostic ion at *m/z* 510
203 (S4E Fig) identified a Le^y hexasaccharide [21].
204

205 **SadP subtype P_N and P_O binding to purified glycolipids**

206 TLC overlay assay is a powerful method to analyze glycolipid receptor function. The carbohydrate-
207 binding specificity of SadP to globo series isoreceptors was determined using purified glycolipids (all TLC
208 binding results are summarized in Table 1). Type P_N bound preferentially to Gb3 and P1 glycolipid, and also
209 weakly to Gb4 and the Forssman glycolipid (Fig 2C). SadP type P_O also bound strongly to Gb3 and P1, i.e. to
210 glycolipids with terminal Galα1–4Gal, whereas there was no binding to Gb4 and the Forssman glycolipid (Fig
211 2D). There was no binding to the non-acid glycolipid fraction from the pig intestine, containing the Globo H.
212 The type P_N SadP binding to dilutions of globo series glycolipids was analyzed with TLC overlay assay
213 to the detection limit for the various glycolipids (S5A Fig). The full length adhesin bound mainly to P1
214 glycolipid, with a detection limit at 0.4 μg, whereas the detection limit for the Gb3 glycolipid was 0.8 μg. The
215 detection limit of SadP(31-328) *N*-terminal domain was 0.2 μg for both Gb3 and P1.
216 Next, the SadP binding to 250 ng of glycolipids immobilized in plastic microtiter wells was
217 quantitatively evaluated by solid phase binding assay (S5B Fig). No binding to the Gb2 glycolipid,
218 galabiosylceramide was detected, whereas there was weak binding to Galα1–4Galβ1–O-*bis*-$(SO_2\text{-}C_{16}H_{33})_2$.
219 SadP was found to bind stronger to Gb3 with non-hydroxy ceramide than to Gb3 with hydroxy ceramide.
220 There was also binding to Gb4 and iGb3 isoglobotriaosylceramide. Binding to P1 pentaosylceramide was at
221 the same level as binding to Gb3 with non-hydroxy ceramide. The difference in the binding to iGb3 (Galα1–
222 3Gal- containing glycolipid) in the microtiter well assay compared to TLC overlay assay could be due to the
223 difference in the presentation of the binding epitope and the clustering of the glycolipid moieties.
224

225 **Galα1–4Gal-dependent endothelial cell binding activity of *S. suis* SadP**

226 *S. suis* type P_N WT strain D282 and the corresponding mutant strain D282Δ*sadP,* containing an insertion
227 mutation into SadP gene, were compared for their binding activity to cultured cell line EA.hy926, which is a
228 primary human umbilical vein cell line fused with a thioguanine-resistant clone of A549 (human lung
229 adenocarcinoma cell line). In addition, binding of purified fluorescently labelled SadP was analysed for the
230 binding to EA.hy926 cells. The cells were grown into glass coverslips for 48 h and the bacteria grown into
231 early log phase were added to cells and were let to adhere for 1 h. After washing and staining with DiffQuick
232 the bacteria were enumerated. The average adherence of wild-type bacteria was calculated to be 8.7 ± 0.5
233 bacteria/field, whereas the mean adhesion of 1.3 ± 0.5 of the insertion mutant Δ*sadP* was significantly less
234 (unpaired t test > 0.0001) (Fig 3A). In addition, the binding of the WT strain was inhibited with 10 μg/ml
235 pigeon ovomucoid, which contains *N*-linked glycans with terminal Galα1–4Gal [23]. The inhibitor reduced
236 the adherence of *S. suis* bacteria to the cells to 0.7 ± 0.1 bacteria/field.

237 For flow cytometry assay, the cells were grown in wells, washed and tested for the binding of FITC
238 labelled SadP. After washing, the cells were harvested with scraping and analyzed with flow cytometer. The
239 flow cytometry results (dot blot and the histogram) are presented in Figure 3BCD. The median SadP binding
240 with and without inhibitor (pigeon ovomucoid, 10 μg/ml) were 56.2 and 14.9 per 10 000 total events (median
241 of untreated cells was 12.8).

242

243 **Fine specificities toward Gb3 and Gb4 oligosaccharide structures**

244 Next, the ability of the type P_N SadP-D282(31-328) and P_O SadP-6407(31-328) recombinant adhesin
245 constructs to recognize galabiose glycoconjugates was analyzed using isothermal titration calorimetry (Fig
246 4AB). Both type P_N and P_O adhesins were titrated with oligosaccharides representing the terminal epitopes of
247 Gb3 and Gb4, the TMSEt (2-trimethylsilylethyl) glycosides of Galα1–4Gal and GalNAcβ1–3Galα1–4Gal
248 [24]. The dissociation constant values (K_D) of type P_N SadP-D282(31-328) and type P_O SadP-6407(31-328)
249 interaction with Galα1–4Gal were 13.0 ± 1.5 μM (n=0.85, ΔH=-69.0±2.0 and -TΔS=41.1) and 3.5 ± 1.6 μM
250 (n=0.85, ΔH=-40.0±2.7 and -TΔS=8.8) respectively. The K_D for interaction of P_N and P_O adhesins with

251 GalNAcβ1–3Galα1–4Gal compared to Galα1–4Gal were 2.6-fold higher with type P_N (K_D= 33.7±3.5 μM,
252 n=1.14, ΔH=-40.1±2.0 and -TΔS=14.0) and 269-higher with type P_O (K_D= 940±680 μM, n=0.13, ΔH=-
253 197±5500 and -TΔS=179).

254 Then, both adhesins were analyzed with AlphaScreen competitive inhibition assay (Fig 4C) for their
255 interaction with Galα1–4Gal in comparison to a glycomimetic inhibitor 3'-phenylurea-derivative (3'-
256 phenylurea-Galα1–4Gal β1–methoxyphenyl), which is a low nanomolar inhibitor of type P_N SadP [19,25].
257 The binding of His-tagged recombinant adhesins to biotinylated pigeon ovomucoid was inhibited with the
258 above oligosaccharides. The IC_{50} values of type P_N adhesin for Galα1–4Galβ1–methoxyphenyl and 3'-
259 phenylurea-Galα1–4Gal β1–methoxyphenyl were 1 μM and 0.030 μM, whereas with type P_O adhesin the
260 corresponding values were 0.3 μM and 70 μM respectively. Also 4 other recombinant type P_O SadP N-
261 terminal-domains were tested using 10 μM inhibitor concentrations (Fig 4D). For all type P_O adhesins the
262 inhibition obtained with the C3'-phenylurea-derivative was weaker than the inhibition obtained with Galα1–
263 4Gal.

264

265 **Site-specific mutation changes the specificity of type P_N recombinant adhesin to type P_O**

266 The binding mechanism of type P_N and P_O SadP was further studied by site-directed mutagenesis. Type
267 P_N adhesin SadP-D282(125-329) was mutated and the mutants were analyzed for the binding to galabiose
268 oligosaccharides with AlphaScreen competitive inhibition assay and ITC. Multiple alignment of SadP
269 homologues allowed us to predict amino acids from the galabiose-binding region that differed between type
270 P_N and P_O (Fig 1A). Based on that, the site-specific amino acid change N285D and E292Q, deletion Δ244-246
271 and N285D&Δ244-246 mutants were constructed into the type P_N SadP(125-328) cloned into the plasmid
272 pET28a. In addition, the 28 amino acid long region H216-D246 of the type P_N was replaced by the
273 corresponding region of type P_O.

274 The WT SadP and mutants were expressed and purified as described in the methods and analysed with
275 SDS-PAGE (S6 Fig). The galabiose-binding strengths of the above mutant constructs, full length SadP, SadP
276 *N*-terminal constructs SadP(31-328), SadP(125-328) and as a negative control site-specific mutant SadP(31-

277 328)W258A, were compared by AlphaScreen asssay (S7 Fig). The binding of full-length SadP to biotinylated
278 pigeon ovomucoid was weaker compared to the *N*-terminal domain constructs, which could be due to a larger
279 size of the full length SadP. The size of the protein could increase the distance of the acceptor and donor beads,
280 and hence reduce the transmission of the singlet oxygen during the assay [19]. As a negative control for the
281 assay, there was no binding of site-specific mutant W258A to pigeon ovomucoid. Of the mutants in the type
282 P_N SadP(125-328) background, mutants N285D and E292Q bound well whereas the binding of mutants Δ244-
283 246 and H216-D246delinsDELFNRFPNKVDSTNNGDGAPFRFFNKE was reduced. The binding of
284 N285D&Δ244-246 mutant was abolished.

285 Next the specificities of mutants were analyzed with AlphaScreen assay using phenylurea-derivative
286 and Galα1–4Gal (S7B - F Figs). All mutants were equally inhibited by the Galα1–4Gal oligosaccharide, but
287 only with the site-specific mutant N285D the inhibitory power of Phenylurea-Galα1–4Gal was weaker
288 compared to Galα1–4Gal, suggesting a specific switch from type P_N to P_O specificity.

289 Next, the Alphascreen inhibition assay was used to compare the effect of site-specific mutation in
290 N285D to the specificity between Galα1–4Gal (terminal Gb3 structure) and GalNAcβ1–3Galα1–4Gal
291 (terminal Gb4 structure) TMSEt glycosides. SadP(125-329) type P_N and site-specific mutant N285D were
292 similarly inhibited with Galα1–4Gal, whereas N285D was not inhibited with even higher concentrations of
293 GalNAcβ1–3Galα1–4Gal (Fig 5AB).

294 The K_D values of type P_N SadP(125-328) and site-specific mutant N285D for oligosaccharides was
295 analyzed with isothermal titration calorimetry (Fig 5C). The wild type SadP(125-329) K_D values for Galα1–
296 4Gal and GalNAcβ1–3Galα1–4Gal were 3.4±0.09 (n=0.76, ΔH=92.0±0.3 and -TΔS=60.8) and 36.0±4.8 μM
297 (n=0.56, ΔH=70.2±5.0 and -TΔS=44.7) respectively. The K_D of site specific mutant N285D with Galα1–4Gal
298 was 22.7±7.2 μM (n=0.52, ΔH=26.8±5.4 and -TΔS=20.5) (6-fold increase compared to wild-type SadP),
299 whereas there was no interaction between N285D and GalNAcβ1–3Galα1–4Gal. Thus, the above results
300 suggest that N285 has a specific role in SadP type P_N binding to GalNAcβ1–3Galα1–4Gal.

301 The effect of N285D mutation to SadP binding to glycolipids incorporated into POPC and POPC-
302 cholesterol liposomes was analyzed by dotting liposomes to PVDF membrane and binding of his-tagged
303 adhesins to immobilized liposomes (Fig 6). WT SadP type P_N bound to Gb4 of both non-cholesterol and

304 cholesterol-containing liposomes, whereas there was no binding by N285D mutant. The acyl chain length or
305 hydroxylation of ceramide had no major effects for Gb3 binding.
306

307 **Structural comparison of recombinant SadP type P_N and its N285D mutant**

308 The site-specific mutant SadP(125-329)N285D was crystallized with five molecules in the
309 crystallographic asymmetric unit (Fig 7A, Table S1). All molecules are similar with subtle differences as
310 suggested by the low root mean square deviation (r.m.s.d) between them (0.25-0.31 Å). The structure of the
311 N285D mutant is similar to that of the native SadP (PDB id 5BOB) with r.m.s.d. of 0.26 Å for 199 aligned
312 residues. The structure consists of three α-helixes and ten β-strands (β1-β10) that form a β-sandwich core
313 domain (Fig 7B). The first β-sheet of the β-sandwich is formed by antiparallel β1-β10-β9-β4-β7-β6 strands
314 and the second β-sheet by β2-β3-β8-β5 in antiparallel fashion.
315 Structural superposition with the Galα1–4Gal – Fhc structure revealed small differences (Fig 7C). The
316 side chain of D285 was found slightly rotated compared to the N285 side chain in the wild type.
317

318 **Resolution of the interaction mechanisms of SadP to globo series glycolipids and C3'-**
319 **Phenylurea–Galα1–4Gal**

320 The key amino acids of both types P_N and P_O are conserved. Y198, Q255, R238 and G233 forms
321 hydrogen bonds with the HO–3', HO-4' and HO-6' of the α'-Gal and the K316 with HO-2 and HO-3 of the β-
322 Gal pyranose ring. W258 most likely forms CH-π interactions with the hydrophobic face of the disaccharide
323 (Fig 7C). Type P_N SadP N285 is on the edge of the binding pocket, and it potentially interacts with the terminal
324 GalNAcβ1–3 of Gb4 or 3-phenylurea-group. To study the SadP interaction with the receptor saccharide
325 Galα1–4Galβ1–4Glc in solution, STD-NMR was applied using type P_N SadP(125-329) and the corresponding
326 mutant N285D (Fig 7D). STD-NMR results suggest that both the type P_N SadP and the mutant N285D interact
327 mainly with the two terminal galactose units of Gb3. With the mutant N285D the interactions of H-4′ and H-
328 4″ are, however, somewhat weaker compared to the interaction of H-6″. Additionally, the mutant N285D does

329 not seem to have a clear interaction with the β-glucoside unit at the reducing end of Gb3, whereas the type P_N
330 SadP appears to have interactions with H-2, H-3 and H-4.

331 In order to find a plausible hypothesis explaining the binding preferences of the galabiose, GalNAcβ1–
332 3Galα1–4Gal and phenylurea-galabioside for WT and the N285D mutant, molecular dynamic simulations of
333 their respective complexes were performed. Briefly, the ligand structures were built without the TMSEt
334 aglycon to simplify the simulations and were then placed in the binding site of SadP (pdb id 5BOA) with the
335 Galα1–4Gal disaccharide oriented as in the crystal structure. The terminal GalNAcβ1 residue was oriented
336 with the dihedral glycosidic bond angles minimized with the H1 parallel to H4' of Galα1–4Gal. The three
337 complexes with WT were mutated by exchanging N285 for D285. The complexes were unrestrainedly
338 subjected to 100 ns molecular dynamics simulations and all starting conformations converged towards similar
339 stable complex geometries (Fig 8A-E), except for the phenylurea derivative in complex with the N285D mutant
340 for which light constraints on the backbone atoms of protein secondary structures were applied (Fig 8F). The
341 α-face of the β-galactoside residue of all three ligands stacked to W258 forming CH-π interactions and the
342 galabiose disaccharide moiety in all simulations retained the interactions and hydrogen bonds observed in the
343 X-ray structure with the Gb3 trisaccharide (PDB id 5boa). The HO-4' of galabiose did not interact directly
344 with N285, but instead via N285 sidechain NH hydrogen bond donation to a network of hydrogen bonds
345 involving 3-5 water molecules and the G233 carbonyl oxygen (Fig 8A and S9 movie). Mutation of N285 to
346 D285 influenced the directionalities of the hydrogen bonding partners in this network (Fig 8D and S10 movie),
347 which may explain the different STD-NMR data with WT and the D285 mutant. The simulations with the
348 GalNAcβ1–3Galα1–4Gal and the phenylurea-galabioside in complex with the WT protein converged to
349 geometries that revealed a highly populated interaction between the ligands and N285. The ring oxygen of the
350 GalNAc residue of the trisaccharide and the urea carbonyl oxygen of the phenylurea derivative accepted
351 hydrogen bonds from the N285 side chain NH_2 (Fig 8B-C and S11 - S12 movies). The GalNAc HO-4 of the
352 trisaccharide formed transient hydrogen bonds to side chain amide groups of E286 and E234, while the phenyl
353 ring of the phenylurea ligand did not form any persistent interactions with any protein residue. The highly
354 populated hydrogen bonds of the trisaccharide GalNAc ring oxygen and the phenylurea carbonyl oxygen to
355 N285 could not form in the N285D mutant (Fig E-F and S13-14 movies). Instead, it was disrupted by the

356 formation of a water network between the D285 side chain carboxylate and the GalNAc ring oxygen of the
357 trisaccharide (Fig 8E), while the phenylurea moiety of the phenylurea galabioside rotated away from the D285
358 side chain carboxylate (Fig 8F). This lack of direct hydrogen bond interactions of the GalNAc ring oxygen
359 and urea carbonyl oxygen may at least partly explain the less efficient binding of the trisaccharide and phenyl
360 urea derivative to the N285D mutant.
361

362 **Discussion**

363 SadP carbohydrate specificity to neutral glycolipids from lungs was studied by TLC overlay assay. The
364 carbohydrate receptors for *S. suis* adhesion in respiratory tract and lungs have remained elusive. We studied
365 here how SadP type P_N and P_O recognize glycolipids isolated from pig lung. The major pig lung neutral
366 glycolipids were identified as Gb3, Gb4, the H type 2 pentaosylceramide, the Galili pentaosylceramide and
367 Le^b and Le^y hexaosylceramides (Fig 2). In TLC overlay assay, type P_N SadP bound to Gb3 and Gb4, whereas
368 type P_O SadP bound only to Gb3. Both SadP types showed no binding to Galα1–3Gal-containing Galili
369 pentaosylceramide. (Fig 2AB, Table 1). Therefore, our findings suggest that globo series glycolipids are the
370 major carbohydrate receptors for *S. suis* in pig lung. Although fucose-binding lectins have been described both
371 in bacterial and fungal lung pathogens, no fucose-specific binding activity was found in SadP. The globo series
372 glycolipids have been shown to be widely expressed in many tissues of pigs [26]; hence, they can potentially
373 serve as SadP receptors for other host organs and brain capillary veins critical for *S. suis* invasive infection.
374 We show that the binding of *S. suis* serotype 2 *sadP*-mutant to endothelial cell line was significantly reduced
375 and binding of both the WT *S. suis* bacteria and recombinant SadP adhesin were inhibited with the galabiose-
376 containing pigeon ovomucoid.

377 The oligosaccharide inhibition assays performed with both whole bacteria and recombinant adhesin
378 suggest that the reducing end β-Glc of Gb3 is required for optimal binding [16,19]. The TLC overlay assay
379 using purified glycolipids showed that SadP bound strongest to the P1 glycolipid (Fig 2BC and S5A Fig). The
380 preferential binding to the P1 glycolipid might also be due to a more optimal presentation of the epitope on
381 the longer core chain, whereas hindered presentation of binding epitope in galabiosyl ceramide might explain,
382 why SadP did not bind to Gb2 in TLC overlay assay. The presence of P1 structure is not well known in pig,

383 however it was found in α1,3GalT knockout pig kidney, since this glycosyltransferase is a direct competitor
384 of α1,4GalT [27]. This might be important for surveillance of pathogens in xenotransplantation.

385 The fine specificity of SadP subtypes was further analyzed with ITC and competitive inhibition assay.
386 The ratio of K_D values (Galα1–4Gal / GalNAcβ1–3Galα1–4Gal) for type P_N SadP was 0.4 whereas for type
387 P_O it was 0.004, which is in accordance to the corresponding hemagglutination inhibition MIC values observed
388 with bacterial hemagglutination assays [16]. Moreover, the IC_{50} values for Galα1–4Gal and 3'-phenylurea-
389 Galα1–4Gal are in accordance to previous hemagglutination inhibition MIC concentrations for type P_N and P_O
390 *S. suis* [25].

391 The mechanism of how the two SadP subtypes differ in their recognition of Gb3, Gb4 and glycomimetic
392 3-phenylurea-galabiose has not been studied before. The multiple-sequence alignment of SadP showed that
393 specific amino acids G233, E249 and N285 of type P_N in galabiose-binding domain are different compared to
394 type P_O SadP (Fig 1A). Of these, only substitution from asparagine to aspartate at position 285 is found in all
395 hemagglutinating type P_O adhesins. To analyze the mechanisms how SadP type P_N can bind to Gb4 terminal
396 trisaccharide (GalNAcβ1–3Galα1–4Gal) and 3'-phenylurea-galabiose, we designed mutations to type P_N SadP
397 and analyzed their binding to galabiose. The SadP type P_N region from H216 to D246 was swapped to the
398 corresponding region of type P_O (to contain substitution from amino acid G to N at position 233). The binding
399 of this mutant to galabiose was reduced compared to the wild type adhesin (S6A Fig), but was still sufficient
400 to further analyze the oligosaccharide specificity. Deletion Δ244-246 and site-specific mutations N285D and
401 E292Q bound also to galabiose at sufficient level (S6A Fig) allowing testing with 3'-phenylurea-derivative to
402 compare for type-specificity. Of these mutants, only N285D lost the ability to be efficiently inhibited by 3'-
403 phenylurea-derivative (S6B-F Fig), suggesting that N285 has an important role in interacting with the
404 phenylurea-group. The amino acid region 216-246 including G233N substitution have possibly no significant
405 role alone in the interaction with HO-3' substituted phenylurea-galabiose. However, G233 could still
406 synergistically interact with phenylurea. Therefore, the N285D mutant was chosen for further studies for the
407 type P_N binding mechanism. The WT and N285D mutant were compared for their binding to the soluble Gb4
408 terminal trisaccharide GalNAcβ1–3Galα1–4Gal and to globo series glycolipids Gb3 and Gb4 (Figs 5 and 6).

409 The results show consistently that N285 plays a specific role in Gb4 binding as demonstrated by competitive
410 inhibition assay, ITC and liposome binding assay.

411 Molecular dynamics calculations were performed with the structures representing type P_N SadP(125-
412 329) and its N285D site-specific mutant. Both proteins were analyzed with Galα1–4Gal, GalNAcβ1–3Galα1–
413 4Gal and 3'-phenylurea-Galα1–4Gal. In accordance to the co-crystal structure (PDB id. 5boa), the galabiose
414 HO-4', HO-6', HO-2 and HO-3 were hydrogen bonded to Y198, G233, R238, Q255 and K316. Hydrophobic
415 interaction with the α-face of the β-galactoside stacked to W258 forming CH-π interactions. The above
416 interactions were conserved in WT and N285 mutant. WT SadP N285 formed hydrogen bonds to GalNAc O-
417 5 ring oxygen of GalNAcβ1–3Galα1–4Gal and to urea's carbonyl group (Fig 8B). The amino acid D285 of
418 site-pecific mutant formed hydrogen bonds with solvent water molecules when simulated with GalNAcβ1–
419 3Galα1–4Gal and 3'-phenylurea-Galα1–4Gal. These results together with the AlphaScreen and ITC data give
420 us a plausible explanation for the molecular mechanism of how type P_N SadP interact with Gb4 terminal
421 GalNAc and with phenylurea-galabiose derivative. In addition, WT SadP amino acid G233 interacts with the
422 terminal GalNAc of Gb4 via hydrogen bond to N-Acetyl-group or to urea's -NH group (Fig 8C).

423 SadP does not have any sequence homologs in other galabiose-recognizing proteins, however
424 structurally *E. coli* PapG and SadP galabiose-binding sites share some similarities. They both contain a β-sheet
425 composed of antiparallel β-strands, which are adjacent to the galabiose-binding domain (PDB; *E. coli* 1j8r, *S.*
426 *suis* 5boa). In SadP, the binding site is formed by the alpha-helix and a non-helix loop, whereas in PapG the
427 galabiose binding site is located in the middle of two non-helix loops. Other galabiose binding proteins such
428 as *P. aeruginosa* LecA (4yw6), *E. coli* verotoxin (1bos) and *Lyophyllum decastes* fungal lectin (4ndv) show
429 different combining site architectures. As previously suggested, the galabiose-binding adhesin SadP from
430 gram-positive and *E. coli* P fimbrial adhesins from gram-negative bacteria are an example of convergent
431 evolution towards binding to the galabiose oligosaccharide. The structural similarities thus confirm this
432 hypothesis.

433 Hemagglutination positive strains expressing type P_N SadP from pig and zoonotic meningitis strains
434 belong to serotype 2 and clonal group CC1 [6,17]. *S. suis* hemagglutination type P_O strains were mostly found
435 from serotype 4 and were isolated from lungs of strains causing pneumonia [28]. The majority of strains that

436 have the gene encoding type P_N SadP belongs to a distinct population consisting of systemic isolates, which
437 were identified in a large BRaTP1T consortium functional genomic study [29]. *S. suis* strains that cause human
438 meningitis comprise a separate clade and are thought to have evolved when the pig farming was intensified in
439 1920s [29]. Zoonotic *S. suis* strains as a specific population have been thought to exponentially spread to
440 different geographical areas due to the selection of pigs that are optimal in terms of productivity. The BRaTP1T
441 consortium study of *S. suis* genomic signatures for pig and human infections has suggested that the systemic
442 infections are genetically determined and the genome size of highly virulent strains is reduced and enriched
443 with virulence genes. Since the type P_N SadP adhesin gene has not been lost in strains causing systemic
444 infections, it is tempting to speculate that the recognition of Gb4 may play a specific role in invasive infections.
445 In a previous study by Ferrando et al. (2017), type P_N SadP (designated SadP1) present in CC1 clonal group
446 of *S. suis* strains [17] was found to specifically adhere better to human intestinal cells (Caco-2) than to pig
447 intestinal cells. This could indicate that type P_N SadP has specific binding preferences to human intestine, thus
448 promoting zoonosis.

449 The results of the present study could have implications toward the therapeutic applications targeting
450 SadP and *S. suis* systemic infections (summarized in Fig 9). The gene encoding SadP type P_N has a downstream
451 homolog, which is a pseudogene (has a stop codon). It could be speculated, that the gene encoding SadP type
452 P_N is a putatively duplicated gene, which has evolved to bind Gb4 (Figure 9A). This study shows the molecular
453 mechanism how N285 contributes to the binding of terminal GalNAcβ1- saccharide of Gb4 by a specific
454 interaction with GalNAc hexose ring oxygen (Figure 9BC). The analogous N285-mediated binding
455 mechanisms to Gb4 and glycomimetic phenylurea-galabiose could be utilized in the design of therapeutics
456 against the invasive systemic infections without disturbing the commensal *S. suis* bacteria colonizing pig upper
457 respiratory tract.

458

459 # Materials and Methods

460 ## Bacterial strains

461 The chromosomal DNA was isolated from type P_N and P_O *S. suis* strains as described before [6] using
462 GelElute bacterial genomic DNA kit according to the manufacturer's instructions. *Streptococci* were grown in
463 Todd Hewitt broth supplemented with 0.5 % yeast extract or in Columbia agar plates supplemented with 5 %
464 sheep blood in 5 % CO_2 at 37°C and *E. coli* strain were grown in LB medium. Antibiotics used were 30 µg/ml
465 (pET28) and 100 µg/ml ampicillin pET46EkLIC) in *E. coli* and 500 µg/ml kanamycin for *S. suis* D282Δ*SadP*
466 mutant [6].
467 *Escherichia coli* NovaBlue (Novagen) was used for the cloning of SadP constructs and strain
468 BL21(DE3) for expression of the adhesin.
469

470 ## Cloning and construction of recombinant adhesins

471 The galabiose binding *N*-terminal domains of SadP from hemagglutination subtypes P_N and P_O were
472 cloned into pET46EkLIC vector as follows: The primer pairs SadPDER48
473 (gacgacgacaagatagaatcgctagaaccagatgtt) and SadPDER49 (gaggagaagcccggtttattcttctcaagggtaatctc) were
474 designed to clone the 882 bp fragment of the adhesin *N*-terminal galabiose binding domain. The fragments
475 were amplified with Phusion HotStart II DNA polymerase and were cloned into LIC-vector (LIC, ligation
476 independent cloning) pET46EkLIC (Novagen). The ligation products were transformed into NovaBlue
477 competent cells. This construct yielded a 33.4 kDa 6xHis-tagged fusion protein (SadP(31-328)). The sequence
478 of *N*-terminal domains of SadP homologs were verified by sequencing with T7 promoter and T7 terminator
479 primers. The vectors were transformed into expression strain BL21(DE3).
480 Site-specific mutant SadP(31-328) W258A was constructed by PCR with 5'-phosphorylated forward
481 primer tgg aac gca tct gct ggt caa gct and reverse primer ttg aga att aaa acc att ttc. The PCR product was
482 amplified with Phusion HotStart II DNA polymerase and the PCR fragment was ligated with T4 ligase
483 (Promega) and transformed into Novablue competent cells. The resulting plasmid SadP(31-328)W258A-
484 pET28a was sequenced to verify the mutation.

485 Synthetic gene construct and the corresponding site-specific mutants for WT SadP(125-328) type P_N
486 were obtained from Genscript. Site-specific mutants constructed were Δ244-246 (PSAD-1), N285D (PSAD-
487 2), E292Q (PSAD-3), N285D&Δ244-246 (PSAD-4) and H216-
488 D246delinsDELFNRFPNKVDSTNNGDGAPFRFFNKE (PSAD-5) (replacement of aa 216-242 into
489 corresponding sequence of type P_O of strain 6107).
490

491 **Bioinformatics**

492 To compare the distribution of SadP alleles in *S. suis* from different infections e.g. invasive, respiratory
493 and non-clinical samples, the presence of either P_N or P_O allele was detected from 374 previously published *S.*
494 *suis* isolates [29] using SRST2 software version 0.2.0 with default settings [30]. The SadP genes from *S. suis*
495 P1/7 (AM946016.1) and 6407 (NZ_CP008921.1) were used as reference sequences for P_N and P_O, respectively.
496 The distribution of P_N and P_O alleles in the *S. suis* phylogeny was investigated by creating a core-genome
497 maximum-likelihood tree using Roary version 3.12.0 [31] and RAxML version 8.2.8 [32] from the 374
498 isolates.
499

500 **Expression and purification of SadP proteins**

501 The bacteria were grown at 30°C, 250 rpm, to an OD600 of 0.5, and the protein expression was induced
502 with 0.2 mM IPTG for 3.5 hours. Bacteria were harvested by centrifugation with 3000 x g, at +4°C and stored
503 at -84°C. The recombinant protein was purified with Ni-NTA affinity chromatography. Briefly, bacteria were
504 lysed with 0.4 mg/ml hen egg lysozyme (Sigma) in 50 mM sodium phosphate buffer pH 8.0, containing 0.5 M
505 NaCl, EDTA-free protease inhibitor cocktail (Pierce), 20 mM imidazole, 20 µg/ml deoxyribonuclease and 1
506 mM $MgCl_2$ on ice for 30 mins. The lysate was sonicated to further homogenize the cell debris and was
507 centrifuged at 20000 x g, +4°C for 30 min. The filtered lysate was purified with Ni-NTA affinity
508 chromatography using HiPrep FF column connected to Äktaprime plus, GE Healthcare at +25°C. Further
509 purification was done with a gel filtration HiLoad 16/60 Superdex 200 column using Tris-HCl, pH 7.5, 0.15
510 M NaCl as running buffer. The purity of the recombinant proteins was analyzed with SDS-PAGE

511 chromatography. For native gel electrophoresis, the recombinant proteins were diluted to sample buffer
512 without SDS and were separated with 8 % polyacrylamide gel.
513

514 **Crystallization**

515 SadP N285D mutant (15 mg/ml) was crystallized with the hanging-drop vapor-diffusion method. The
516 well condition contained 1.3-1.5 M sodium citrate tribasic, 0.1 M sodium cacodylate, pH 6.5. Equal volumes
517 of well solution and protein (2 μL+2μL) were mixed and the crystals grew at 16 °C, initially as haystacks of
518 very thin needles. Single crystals suitable for data collection were subsequently grown by using the seeding
519 technique. A small part of the haystack was crushed with a metallic needle in the crystallization drop and 0.2
520 μL were diluted in 20 μL of mother liquor to create a seeding stock. Pre-equilibrated (1-d old) crystallization
521 drops were seeded with 0.1 μL of the seeding stock. Single crystals started to grow after ~3 days.
522

523 **Data collection and crystal structure determination**

524 Diffraction data were collected on the BioMAX beamline at MAX IV (Lund, Sweden) from a single
525 crystal under cryogenic temperatures (100K). The crystal diffracted to 1.85 Å but the resolution was later
526 truncated during processing to 2.05 Å owing to completeness. The crystal was found to belong to the *C*2 space
527 group. Data processing was carried out with automated procedures in EDNA [33] and scaling was done with
528 AIMLESS [34].
529 Molecular replacement with PHASER [35] was carried out to obtain initial phases using the native
530 structure as a template. Five molecules in the crystallographic asymmetric unit were located (Matthews
531 coefficient 2.67 $Å^3$/Da corresponding to ~53.9% solvent content). Initial building of the structure was carried
532 out using the automated building procedure in BUCCANEER [36] using the ccp4i2 interface [37]. After the
533 initial building that produced an almost complete structure, the refinement continued in PHENIX v.1.17.1-
534 3660 [38] using simulated annealing at 1000K with maximum likelihood as target function. The first round of
535 simulated annealing resulted in R_{work}/R_{free} of 0.227/0.269. Inclusion of waters and rounds of manual rebuilding
536 using COOT [39] alternated with PHENIX refinement resulted in the final structure with R_{work}/R_{free} of

537 0.174/0.221 (Table S1). A glycerol molecule was found bound in each of the A, C, and D subunits. Two
538 glycerol molecules were located in the binding site of subunit B. The structure has been deposited to the Protein
539 Data Bank under the accession code 6YRO
540 .
541

542 **Isolation of SadP binding glycosphingolipids from porcine lung**

543 Non-acid glycosphingolipids were isolated from porcine lung as described [40]. Briefly, the lung tissue
544 was lyophilized, and then extracted in two steps in a Soxhlet apparatus with chloroform and methanol (2:1 and
545 1:9, by volume, respectively). The material obtained was subjected to mild alkaline hydrolysis and dialysis,
546 followed by separation on a silicic acid column. Acid and non-acid glycosphingolipid fractions were obtained
547 by chromatography on a DEAE-cellulose column. In order to separate the non-acid glycolipids from alkali-
548 stable phospholipids, this fraction was acetylated and separated on a second silicic acid column, followed by
549 deacetylation and dialysis. Final purifications were done by chromatographies on DEAE-cellulose and silicic
550 acid columns.
551 The total non-acid glycosphingolipid fraction (9 mg) from porcine lung was first separated on an
552 Iatrobeads (latrobeads 6RS-8060; Iatron Laboratories, Tokyo) column (1.0 g) and eluted with increasing
553 volumes of methanol in chloroform. Aliquots of the fractions obtained were analyzed by thin-layer
554 chromatography. Fractions that were coloured green by anisaldehyde were tested for binding of SadP using
555 the chromatogram binding assay. SadP bound to a fraction containing glycosphingolipids migrating in the tri-
556 and tetraglycosylceramide regions on thin-layer chromatograms. This fraction (1.0 mg) was further separated
557 on an Iatrobeads column (1.0 g), eluted with chloroform/methanol/water 65:25:4 (by volume), 30 x 0.5 ml,
558 followed by chloroform/methanol/water 65:25:4, 10 mi. The SadP binding triglycosylceramide was eluted in
559 fractions 12-17, and these fractions were pooled into one fraction migrating as a single band (0.2 mg, denoted
560 fraction PL-1), and fraction migrating as a double band (0.3 mg, denoted fraction PL-2) on thin-layer
561 chromatograms. In addition, a SadP tetraglycosylceramide was present in fractions 19-31, and pooling of
562 these fractions gave 0.4 mg (denoted fraction PL-4).
563

564 **Reference glycosphingolipids**

565 Total acid and non-acid glycosphingolipid fractions were isolated as described [40], and the individual
566 glycosphingolipids were obtained by repeated chromatography on silicic acid columns, and by HPLC, and
567 identified by mass spectrometry [20,41] and 1H NMR spectroscopy [42].
568

569 **Thin-layer chromatography**

570 Aluminum- or glass-backed silica gel 60 high performance thin-layer chromatography plates
571 (Merck, Darmstadt, Germany) were used for thin-layer chromatography, and chromatographed with
572 chloroform/methanol/water (60:35:8 by volume) as solvent system. The different glycosphingolipids
573 were applied to the plates in quantities of 4 μg of pure glycosphingolipids, and 20-40 μg of
574 glycosphingolipid mixtures. Chemical detection was done with anisaldehyde [43].
575

576 **Radiolabeling**

577 Aliquots of 100 μg of the different SadP protein preparations were labeled with ^{125}I by the Iodogen
578 method according to the manufacturer's instructions (Pierce/Thermo Scientific), giving approximately 2000
579 cpm/μg protein.
580
581

582 **Chromatogram binding assays**

583 Binding of radiolabeled proteins to glycosphingolipids on thin-layer chromatograms was done as
584 described [44]. Chromatograms with separated glycosphingolipids were dipped for 1 min in diethylether/n-
585 hexane (1:5, by volume) containing 0.5% (w/v) polyisobutylmethacrylate (Aldrich Chern. Comp. Inc.,
586 Milwaukee, WI). After drying, the chromatograms were soaked in PBS containing 2% (w/v) bovine serum
587 albumin, 0.1% (w/v) NaN_3 and 0.1% (w/v) Tween 20 (Solution A), for 2 h at room temperature. Thereafter
588 the plates were incubated with ^{125}I- labeled SadP protein (l -5 x 10^6 cpm/ml) for 2 h at room temperature. After

589 washing six times with PBS, and drying, the thin-layer plates were autoradiographed for 12 h using XAR-5
590 x-ray films (Eastman Kodak, Rochester, NY).
591

592 **Microtiter well assay**

593 Binding of radiolabeled SadP to glycosphingolipids in microtiter wells was performed as described [44].
594 In short, 250 µg of pure glycosphingolipids in methanol were applied to microtiter wells (Falcon 3911, Becton
595 Dickinson Labware, Oxnard, CA). When the solvent had evaporated, the wells were blocked for 2 h at room
596 temperature with 200 µl of BSA/PBS. Thereafter, the wells were incubated for 4 h at room temperature with
597 50 µl of ^{125}I-labeled SadP (2 x 10^3 cpm/µl) diluted in BSA/PBS. After washing 6 times with PBS, the wells
598 were cut out and the radioactivity was counted in a gamma counter.
599

600 **LC-ESI/MS of native glycosphingolipids**

601 Native glycosphingolipids were analyzed by LC-ESI/MS as described [45]. Glycosphingolipids were
602 dissolved in methanol:acetonitrile in proportion 75:25 (by volume) and separated on a 200x0.150 mm column,
603 packed in-house with 5 µM polyamine II particles (YMC Europe GmbH, Dinslaken, Germany). An
604 autosampler, HTC-PAL (CTC Analytics AG, Zwingen, Switzerland) equipped with a cheminert valve (0.25
605 mm bore) and a 2 µl loop, was used for sample injection. An Agilent 1100 binary pump (Agilent technologies,
606 Palo Alto, CA) delivered a flow of 250 µl/min, which was split down in an 1/16" microvolume-T (0.15 mm
607 bore) (Vici AG International, Schenkon, Switzerland) by a 50 cm x 50 µm i.d. fused silica capillary before the
608 injector of the autosampler, allowing approximately 2-3 µl/min through the column. Samples were eluted with
609 an aqueous gradient (A:100% acetonitrile to B: 10 mM ammonium bicarbonate). The gradient (0-50% B) was
610 eluted for 40 min, followed by a wash step with 100% B, and equilibration of the column for 20 min. The
611 samples were analyzed in negative ion mode on a LTQ linear quadropole ion trap mass spectrometer (Thermo
612 Electron, San José, CA), with an IonMax standard ESI source equipped with a stainless steel needle kept at –
613 3.5 kV. Compressed air was used as nebulizer gas. The heated capillary was kept at 270 °C, and the capillary
614 voltage was –50 kV. Full scan (*m/z* 500-1800, two microscans, maximum 100 ms, target value of 30,000) was

615 performed, followed by data-dependent MS^2 scans (two microscans, maximun 100 ms, target value of 30.000)
616 with normalized collision energy of 35%, isolation window of 2.5 units, activation q= 0.25 and activation time
617 30 ms). The threshold for MS^2 was set to 500 counts. Data acquisition and processing were conducted with
618 Xcalibur software version 2.0.7 (Thermo Fisher Scientific). Manual assignment of glycosphingolipid
619 sequences was done with the assistance of the Glycoworkbench tool (Version 2.1), and by comparison of
620 retention times and MS^2 spectra of reference glycosphingolipids.
621

622 **Endoglycoceramidase digestion and LC-ESI/MS**

623 Endoglycoceramidase II from *Rhodococcus* spp. (Ito) (Takara Bio Europe S.A., Gennevilliers, France)
624 was used for hydrolysis of glycosphingolipids. Briefly, 50 µg of glycosphingolipids were resuspended in 100
625 µl 0.05 M sodium acetate buffer, pH 5.0, containing 120 µg sodium cholate, and sonicated briefly. Thereafter,
626 1 mU of enzyme was added, and the mixture was incubated at 37°C for 48 h. The reaction was stopped by
627 addition of chloroform/methanol/water to the final proportions 8:4:3 (by volume). The oligosaccharide-
628 containing upper phase thus obtained was separated from detergent on a Sep-Pak QMA cartridge (Waters).
629 The eluant containing the oligosaccharides was dried under nitrogen and under vacuum.
630 The glycosphingolipid-derived oligosaccharides were resuspended in 50 µl of water and analyzed by
631 LC-ESI/MS as described [20]. The oligosaccharides were separated on a column (200 x 0.180 mm) packed in-
632 house with 5 µm porous graphite particles (Hypercarb, Thermo-Hypersil, Runcorn, UK). An autosampler,
633 HTC-PAL (CTC Analytics AG, Zwingen, Switzerland) equipped with a cheminert valve (0.25 mm bore) and
634 a 2 µl loop, was used for sample injection. An Agilent 1100 binary pump (Agilent technologies, Palo Alto,
635 CA) delivered a flow of 250 µl/min, which was split down in an 1/16" microvolume-T (0.15 mm bore) (Vici
636 AG International, Schenkon, Switzerland) by a 50 cm x 50 µm i.d. fused silica capillary before the injector of
637 the autosampler, allowing approximately 2-3 µl/min through the column. The oligosaccharides (3 µl) were
638 injected on to the column and eluted with an acetonitrile gradient (A: 10 mM ammonium bicarbonate; B: 10
639 mM ammonium bicarbonate in 80% acetonitrile). The gradient (0-45% B) was eluted for 46 min, followed by

640 a wash step with 100% B, and equilibration of the column for 24 min. A 30 cm x 50 µm i.d. fused silica
641 capillary was used as transfer line to the ion source.

642 The oligosaccharides were analyzed in negative ion mode on an LTQ linear quadrupole ion trap mass
643 spectrometer (Thermo Electron, San José, CA). The IonMax standard ESI source on the LTQ mass
644 spectrometer was equipped with a stainless-steel needle kept at –3.5 kV. Compressed air was used as nebulizer
645 gas. The heated capillary was kept at 270 °C, and the capillary voltage was –50 kV. Full-scan (*m/z* 380-2 000,
646 2 microscans, maximum 100 ms, target value of 30 000) was performed, followed by data dependent MS^2
647 scans of the three most abundant ions in each scan (2 microscans, maximum 100 ms, target value of 10 000).
648 The threshold for MS^2 was set to 500 counts. Normalized collision energy was 35%, and an isolation window
649 of 3 u, an activation q = 0.25, and an activation time of 30 ms, was used. Data acquisition and processing were
650 conducted with Xcalibur software (Version 2.0.7).

651 Manual assignment of glycan sequences was done on the basis of knowledge of mammalian biosynthetic
652 pathways, with the assistance of the Glycoworkbench tool (Version 2.1), and by comparison of retention times
653 and MS^2 spectra of oligosaccharides from reference glycosphingolipids [20].

654

655 **S. suis cell binding assay**

656 EA.hy926 cells were maintained and grown in DMEM, 10 % FCS medium at 37°C, 5 % CO_2. For
657 bacterial binding assay the cells were detached with Trypsin-EDTA, counted and 15 000 cells/well added into
658 round glass coverslips in 24 well-plates. Cells were grown for 24 - 48 h to subconfluency.

659 *S. suis* D282 WT strain and D282-Δ*sadP* were grown in THY overnight at 37°C, 5 % CO_2. Bacteria
660 were diluted 1/20 into prewarmed 37°C THY and were grown to OD550 of 0.2 and were diluted 1/100 into
661 prewarmed DMEM, 10 % FCS without antibiotics. 500 µl of bacterial dilution was pipetted into the wells
662 (MOI 100:1) and the plate was centrifuged with 800 x g, 15 min at 20°C.

663 The plate was incubated at 37°C for 1 h. The wells were washed 4 x 1000 µl of PBS. The cells were
664 stained with DiffQuick kit, washed and the coverslips were mounted on glass slides with Permount.

665 The bacterial binding was quantitated by microscopy with 100 x objective with immersion oil. The
666 bound bacteria were enumerated by counting two wells for each sample. The results were expressed as average
667 of bacteria / optical field.

668 For flow cytometry, N-terminal domain of SadP was labelled with FITC (Sigma-Aldrich) in 0.2 M
669 borate buffer, pH 8.0, 0.16 M NaCl (50:1 molar ratio of FITC/SadP, 83 nmol of SadP and 1.7 µmol of FITC)
670 and the labelling was stopped with Tris-buffer. The labelled SadP was purified from the free FITC with PD-
671 10 (GE Healthcare) desalting column. The EA.hy926 cells were seeded into 6 well culture dishes (1 x 10^5
672 cells/well) and were grown for 48 h. The cells were washed with DMEM and were incubated with 400 ng/ml
673 of labelled SadP with or without 10 µg/ml pigeon ovomucoid. After binding, the wells were washed with
674 phosphate Buffered Saline (PBS, 0.15 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). and were
675 fixed with 2 % (w/v) paraformaldehyde in PBS for 15 min. The cells were washed twice with PBS and the
676 cells were scraped of the wells and suspended into the PBS. 10 000 cells were analyzed for SadP binding with
677 FACSCalibur.

678

679 **Amplified luminescent proximity homogeneous assay (AlphaScreen)**

680 The AlphaScreen assay was optimized as described before [19]. Briefly, the assay was performed using
681 AlphaScreen Streptavidin Donor beads and NiNTA Acceptor beads (PerkinElmer). The molar concentrations
682 of galabiose-containing biotinylated ovomucoid (receptor) and adhesin were optimized by finding the hook
683 point for the interaction by setting up a matrix of proteins in 96 well AlphaPlates (Perkin Elmer) in 10 mM
684 Tris-HCl, pH 7.5, 0.15 M NaCl, 0.2% BSA, 0.05 % Tween 20 0.2% (TBST-0.2%BSA). For the optimized
685 assay dilutions of 5 µl of the His-tagged adhesin and 5 µl of biotinylated ovomucoid were pipetted into the
686 wells and the plates were centrifuged for 1 min, 1000 x g. The plates were incubated at +25°C for 1 hr. This
687 was followed by addition of Ni-NTA- beads (20 µg/ml) and incubation for 1.5 hrs. Finally, streptavidin donor
688 beads were added (20 µg/ml) and the mixtures were incubated for 30 mins and were then measured with the
689 Ensight multimode reader (Perkin Elmer) using excitation wavelength of 680 nm with donor beads and
690 measurement of emission wavelength of 615 nm from AlphaLISA anti-HIS acceptor beads. For inhibition
691 assays the oligosaccharides, whose structures have been described before [25] or depicted in Figure 4 for

692 TMSEt- glycosides of GalNAcβ1–3Galα1–4Gal and 3Galα1–4Gal, were diluted into TBST-0.2%BSA buffer
693 and were mixed with the His-tagged adhesin and biotinylated ovomucoid. The binding was measured as
694 described above. The binding inhibition data was fitted using Prism with settings of log(inhibitor) vs. response
695 slope (four parameters).
696

697 **Isothermal titration calorimetry**

698 SadP-D282(31-328) (P_N), SadP-6107(31-328) (P_O), SadP(125-329) type P_N and site-specific mutant
699 SadP(125-328)N285D were desalted into PBS using PD-10 desalting column (GE Healtcare). The synthetic
700 oligosaccharides were diluted to the same buffer. 0.1 - 0.2 μM of SadP proteins in a 350 μl vessel was titrated
701 with 1.5 - 2 mM solutions of galabiose oligosaccharides using MicroCal (Malvern). The protein solution was
702 stirred with 750 rpm at 25°C and 16 injections of a volume of 2.49 μl were injected at 180 s intervals. The data
703 from single determinations was analyzed with Origin.
704

705 **Preparation of liposomes and SadP binding assay**

706 Vesicles were prepared as described before [46]. Briefly, POPC (1-palmitoyl-2-oleoyl-glycero-3-
707 phosphocholine, Avanti Polar lipids), cholesterol and glycolipids were dissolved and mixed into the
708 chloroform : methanol (2:1, vol/vol) to contain 30 % cholesterol and 2 mM glycolipids. The solvent was
709 evaporated and the lipid film was suspended for 30 min at 60 °C in 10 mM Tris-Cl, 140 mM NaCl buffer. The
710 lipid-buffer suspension was briefly vortexed followed by the extrusion procedure (Avanti mini extruder using
711 0.1 μm polycarbonate membranes filter, (Avanti Polar Lipids, Alabaster, AL, USA)) to form large unilamellar
712 vesicles.
713 PVDF membrane was wetted (wetting in 100 % MeOH, washing 4 x 5 min MilliQ water) and was
714 overlaid onto wet Whatman filter paper. Liposomes were serially diluted 1/10 into Tris-buffered saline and 2
715 μl of liposomes containing 2 mM, 0.2 mM and 0.02 mM of glycolipid was pipetted onto the membrane and
716 the membrane was not allowed to dry during the pipetting. The membrane was saturated with 2 % BSA (w/v)
717 in TBS for 1 h at 20°C and was incubated with 10 μg/ml recombinant SadP for 2 h at 20°C. The membrane

718 was washed 4 x 5 min with TBS and the bound SadP was detected with 1:10000 dilution of anti-His primary
719 antibody (Sigma) and 1:20000 dilution of HRP-labelled secondary rabbit anti-mouse antibody
720 (DakoCytomation). The membrane was incubated with ECL substrate (WesternBright Quantum, Labtech) and
721 the SadP binding was imaged with Fuji LAS-4000.
722

723 **STD-NMR**

724 STD-NMR measurements were carried out on a Bruker AVANCE III HD spectrometer (Bruker BioSpin
725 GmbH, Rheinstetten, Germany) operating at 600.13 MHz, equipped with a nitrogen-cooled triple-channel TCI
726 inverse CryoProbe™. The pulse sequence used for the experiments was the stddiffesgp provided by Bruker.
727 A 1x PBS buffer with a pH of 7.45 with 10% D_2O, containing 50 µM protein and 2500 µM Gb3 trisaccharide
728 was used for the experiments. The on-resonance pulse was set to the methyl groups at 0.9 ppm and the off-
729 resonance pulse was set at 20 ppm. A saturation time of 2 s was used and the experiments were carried out at
730 313 K to somewhat improve the weak signals, likely resulting from slow k_{off}. A blank experiment with only
731 Gb3 and no protein was done to rule out any self-STD.
732

733 **Molecular dynamics simulations**

734 Molecular dynamics simulations were performed with the OPLS3 force field in Desmond (Schrödinger
735 Release 2019-4: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2017;
736 Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2017) using default settings except
737 for the length of the simulation. Simulations were performed with periodic boundary conditions using
738 orthorhombic simulation boxes with SPC water model. Counter ions were used to neutralize each other.
739 Starting conformations of ligands in complex with SadP (pdb id 5BOA) were built by manually placing ligand
740 galabiose residues in an identical position to that in the crystal structure (pdb id 5BOA). Complexes with the
741 mutants were generated by exchanging N285 for D285. The complexes were then subjected to 100 ns
742 molecular dynamics simulations. The complex of the phenylurea derivative with the N285D mutant was not

743 stable during the 100 ns simulations. Therefore, light constraints (1 kcal·mol^{-1}·Å^{-1}) on the protein secondary
744 structures were applied. Molecular images were generated using PyMOL v2.30 (Schrodinger LLC).
745

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750

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752

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Tables

Table 1.

Table 1. Binding of SadP to glycosphingolipids on thin-layer chromatograms

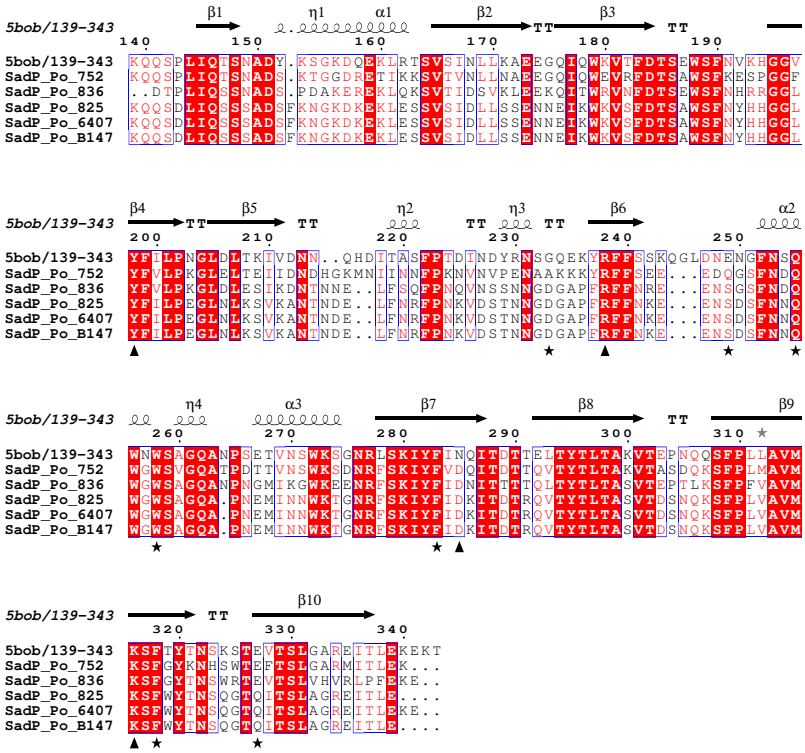
No. Trivial name	Structure	SadP type P_N/P_O	Source
1. Galabiaosylcer (Gb2)	Galα1–4Galβ–O–Cer	-	Human meconium
2. Galabiaosylcer (Gb2-S)	Galα1–4Galβ1–O–*bis*–$(SO_2\text{-}C_{16}H_{33})_2$	-	Synthetic
3. Isoglobotri (iGb3)	Galα1–3Galβ1–4Glcβ1–O–Cer	+/-	Cat intestine
4. Globotri (Gb3)	Galα1–4Galβ1–4Glcβ1–O–Cer	+++/+++	Human erythrocytes
5. Globotetra (Gb4)	GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–O–Cer	+/-	Human erythrocytes
6. Forssman	GalNAcα1–3GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–O–Cer	+/-	Dog intestine
7. para-Forssman	GalNAcβ1–3GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–O–Cer	-	Human erythrocytes
8. P1	Galα1–4Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–O–Cer	+++/+++	Human erythrocytes
9. H type 4/Globo H hexa	Fucα1–2Galβ1–3GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–O–Cer	-	Pig intestine
10. H type 2 penta	Fucα1–2Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–O–Cer	-	Pig lung
11. Galili penta	Galα1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–O–Cer	-	Pig lung
12. Le^b hexa	Fucα1–2Galβ1–3(Fucα1-4)GlcNAcβ1–3Galβ1–4Glcβ1–O–Cer	-	Pig lung
13. Le^y hexa	Fucα1–2Galβ1–4(Fucα1-3)GlcNAcβ1–3Galβ1–4Glcβ1–O–Cer	-	Pig lung
14. A type 2 hexa	Galα1–3(Fucα1–2)Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–O–Cer	-	Pig erythrocytes
15. A type 4/Globo A hepta	GalNAcα1-3(Fucα1-2)Galβ1-3GalNAcβ1–3Galα1-4Galβ1-4Glcβ1–O–Cer	-	Pig intestine

[a] Binding is defined as follows: +++ denotes a highly reproducible binding of SadP type P_N/P_O when 4 μg of the glycosphingolipid was applied on the thin-layer chromatogram, while + denotes an occasional binding, and - denotes no binding even at 4 μg.

Figures

Fig 1.

A.



B

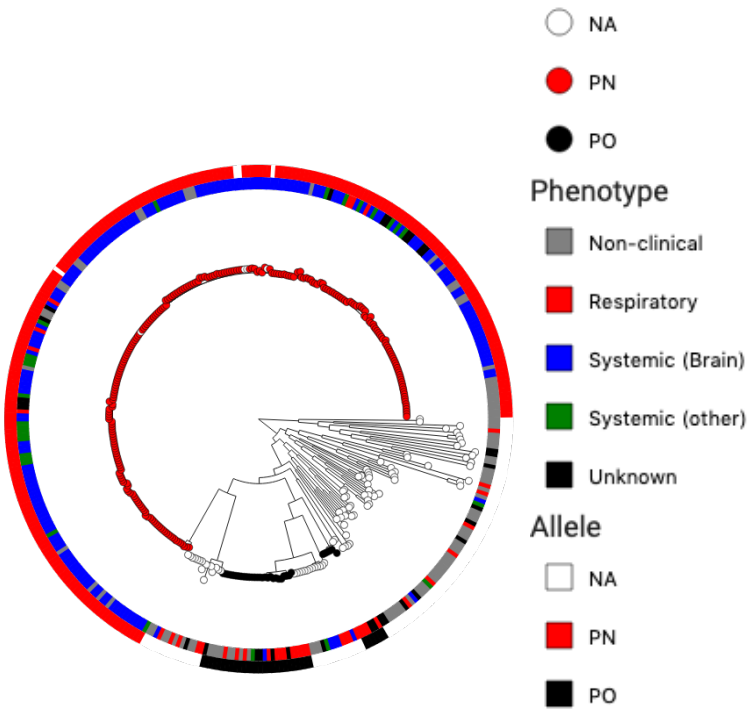


Fig 2.

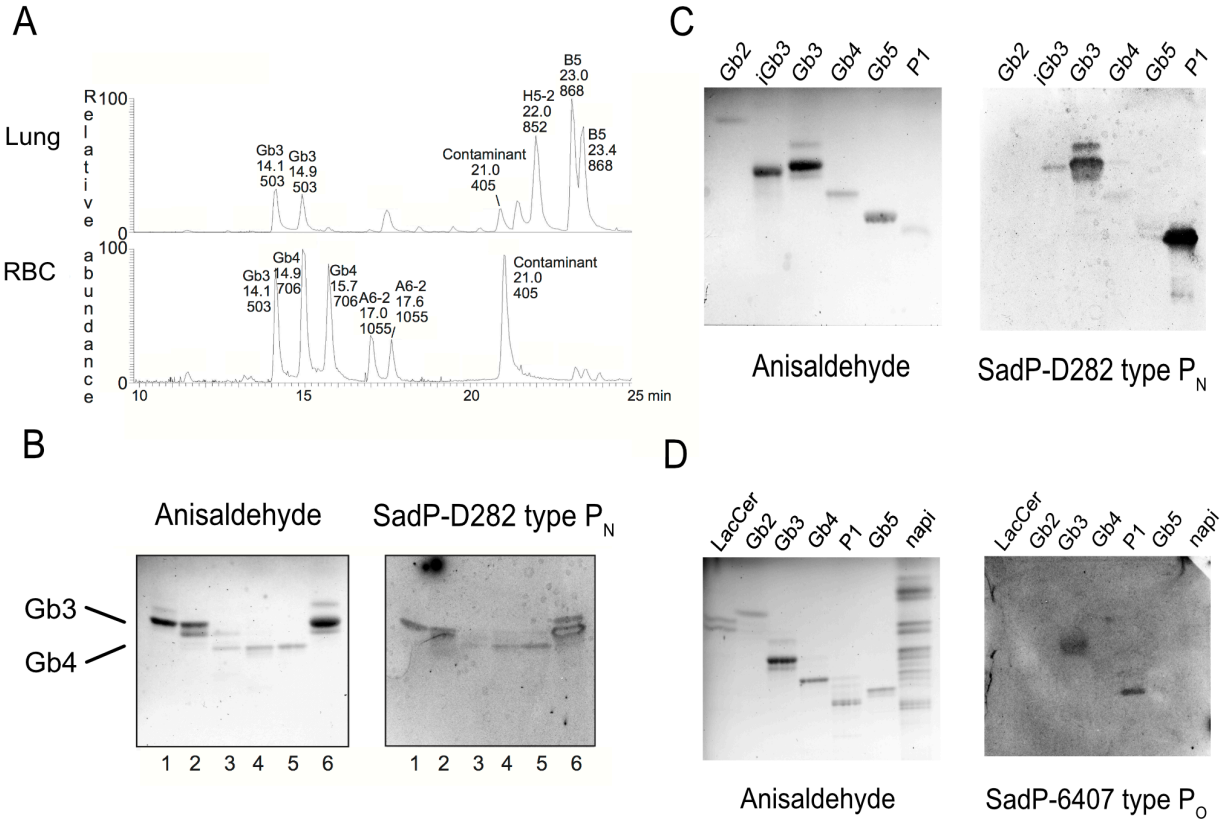

A
Lung
RBC
Relative abundance
100
0
Gb3 14.1 503
Gb3 14.9 503
Contaminant 21.0 405
H5-2 22.0 852
B5 23.0 868
B5 23.4 868
Gb4 14.9 706
Gb4 15.7 706
A6-2 17.0 1055
A6-2 17.6 1055
10
15
20
25 min
B
Anisaldehyde
SadP-D282 type P_N
Gb3
Gb4
1 2 3 4 5 6
C
Gb2 iGb3 Gb3 Gb4 Gb5 P1
Anisaldehyde
SadP-D282 type P_N
D
LacCer Gb2 Gb3 Gb4 P1 Gb5 napi
Anisaldehyde
SadP-6407 type P_O

Fig. 3.

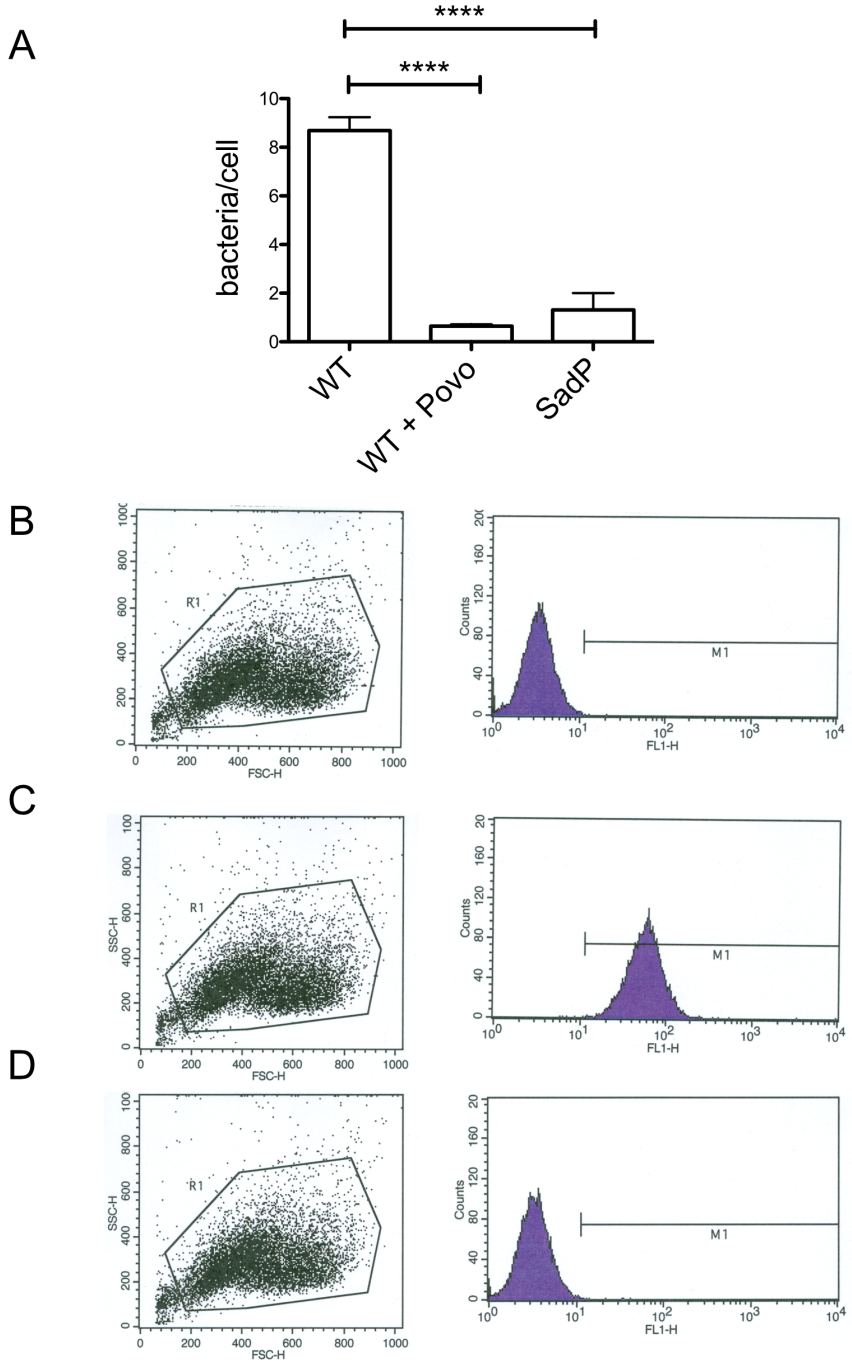

A
****
****
bacteria/cell
10
8
6
4
2
0
WT
WT + Povo
SadP
B
C
D
R1
FSC-H
SSC-H
Counts
M1
FL1-H

Fig 4.

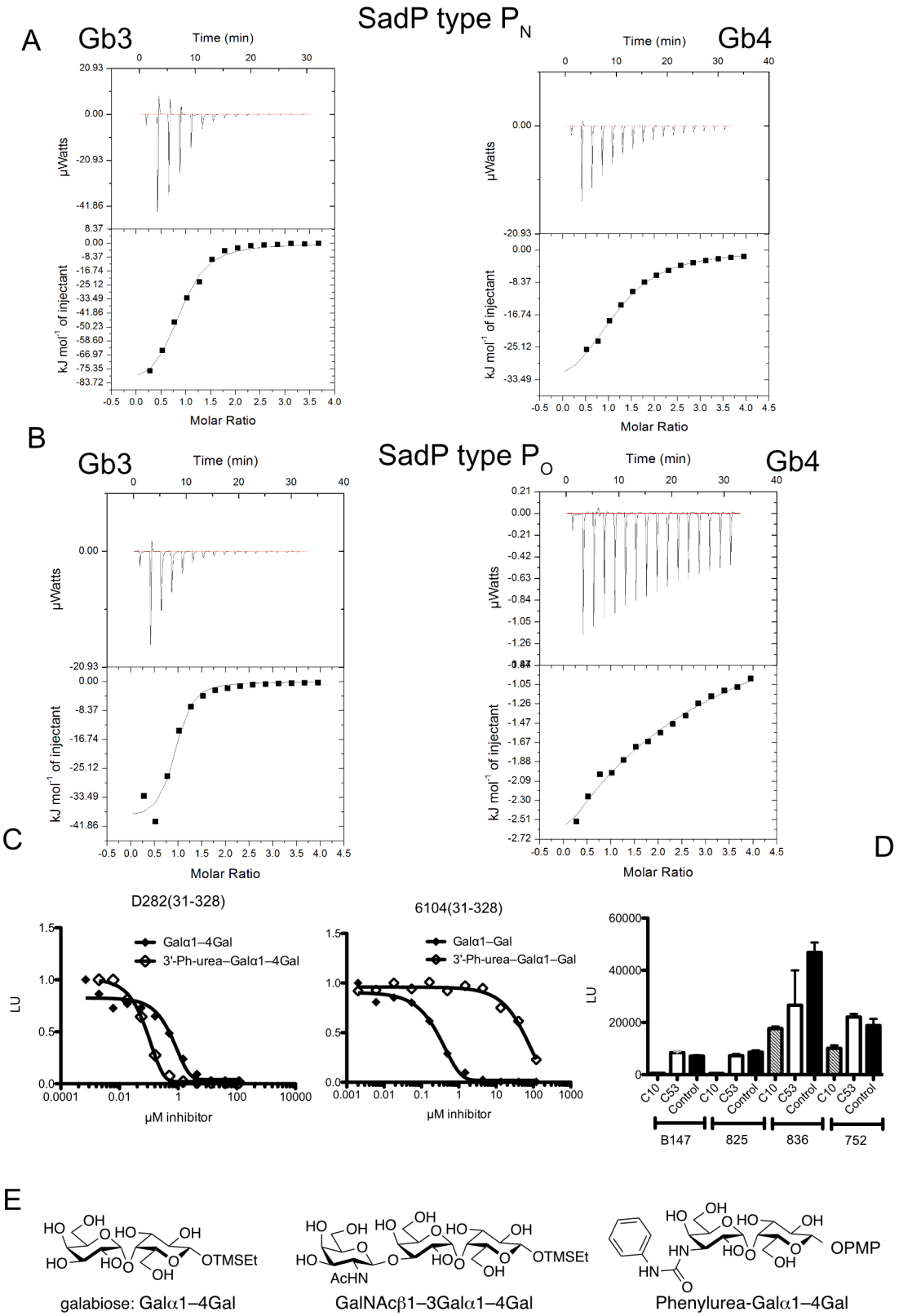

SadP type P_N
A
Gb3
Gb4
Time (min)
µWatts
kJ mol⁻¹ of injectant
Molar Ratio
B
SadP type P_O
C
D
D282(31-328)
6104(31-328)
Galα1–4Gal
3'-Ph-urea–Galα1–4Gal
Galα1–Gal
3'-Ph-urea–Galα1–Gal
LU
µM inhibitor
C10
C53
Control
B147
825
836
752
E
galabiose: Galα1–4Gal
GalNAcβ1–3Galα1–4Gal
Phenylurea-Galα1–4Gal
OTMSEt
OPMP
AcHN

Fig 5.

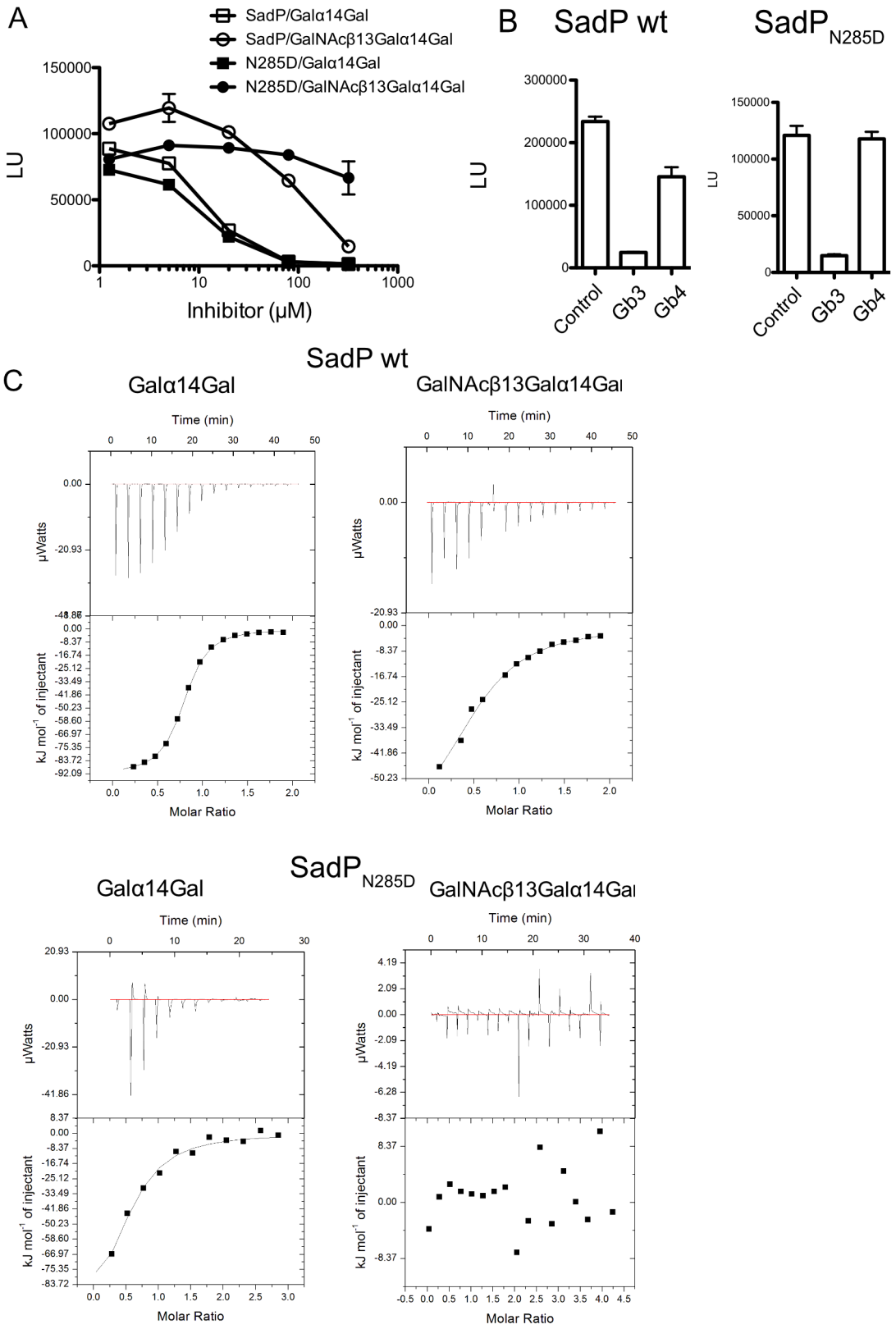

A
SadP/Galα14Gal
SadP/GalNAcβ13Galα14Gal
N285D/Galα14Gal
N285D/GalNAcβ13Galα14Gal
LU
Inhibitor (μM)
B
SadP wt
SadP N285D
Control
Gb3
Gb4
C
SadP wt
Galα14Gal
GalNAcβ13Galα14Gal
SadP N285D
Time (min)
μWatts
kJ mol⁻¹ of injectant
Molar Ratio

Fig 6.

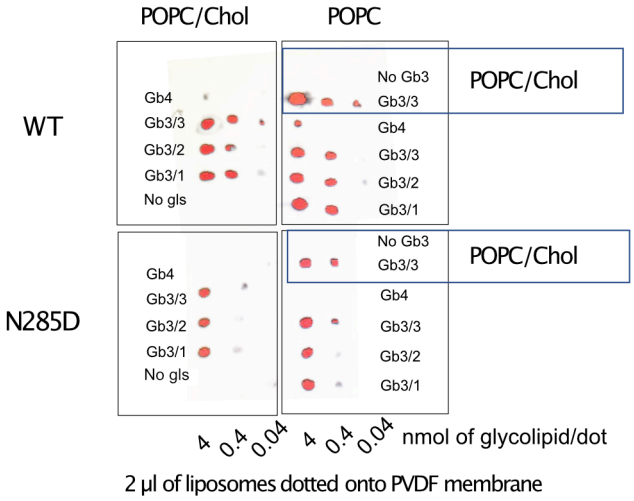

POPC/Chol
POPC
WT
N285D
Gb4
Gb3/3
Gb3/2
Gb3/1
No gls
No Gb3
Gb3/3
Gb4
Gb3/3
Gb3/2
Gb3/1
POPC/Chol
POPC/Chol
4
0.4
0.04
nmol of glycolipid/dot
2 µl of liposomes dotted onto PVDF membrane

Fig 7.

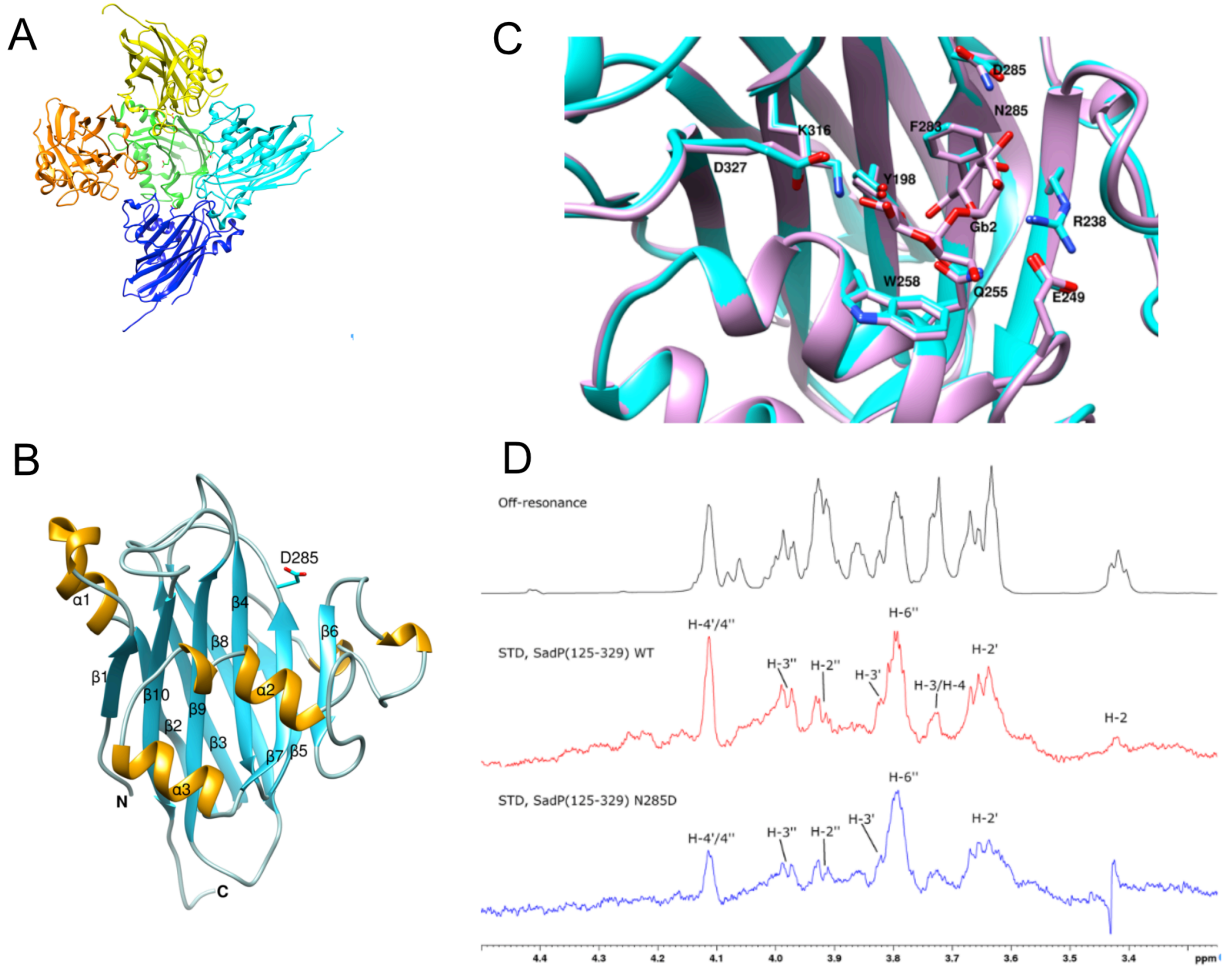

A
B
C
D
D285
α1
β4
β8
β6
β1
β10
β9
α2
β2
β3
β7
β5
α3
N
C
D285
N285
K316
F283
D327
Y198
Gb2
R238
W258
Q255
E249
Off-resonance
STD, SadP(125-329) WT
H-4'/4''
H-3''
H-2''
H-3'
H-6''
H-3/H-4
H-2'
H-2
STD, SadP(125-329) N285D
H-6''
H-4'/4''
H-3''
H-2''
H-3'
H-2'
4.4
4.3
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
ppm

Fig 8.

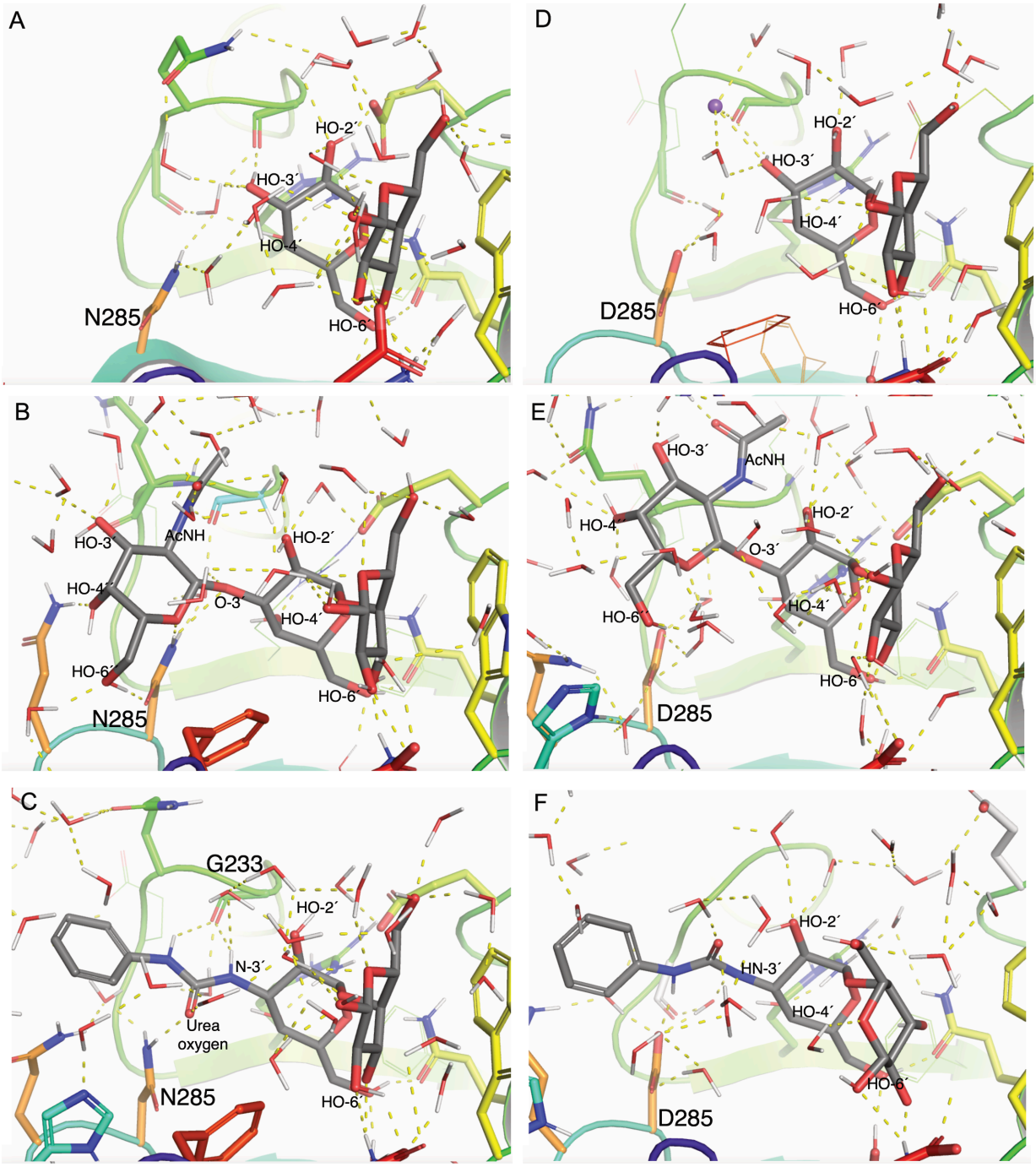

A
HO-2´
HO-3´
HO-4´
N285
HO-6´
D
HO-2´
HO-3´
HO-4´
D285
HO-6´
B
HO-3´
AcNH
HO-2´
HO-4´´
O-3´
HO-4´
HO-6´´
N285
HO-6´
E
HO-3´
AcNH
HO-4´´
HO-2´
O-3´
HO-6´´
HO-4´
HO-6´
D285
C
G233
HO-2´
N-3´
Urea
oxygen
N285
HO-6´
F
HO-2´
HN-3´
HO-4´
HO-6´
D285

Fig 9.

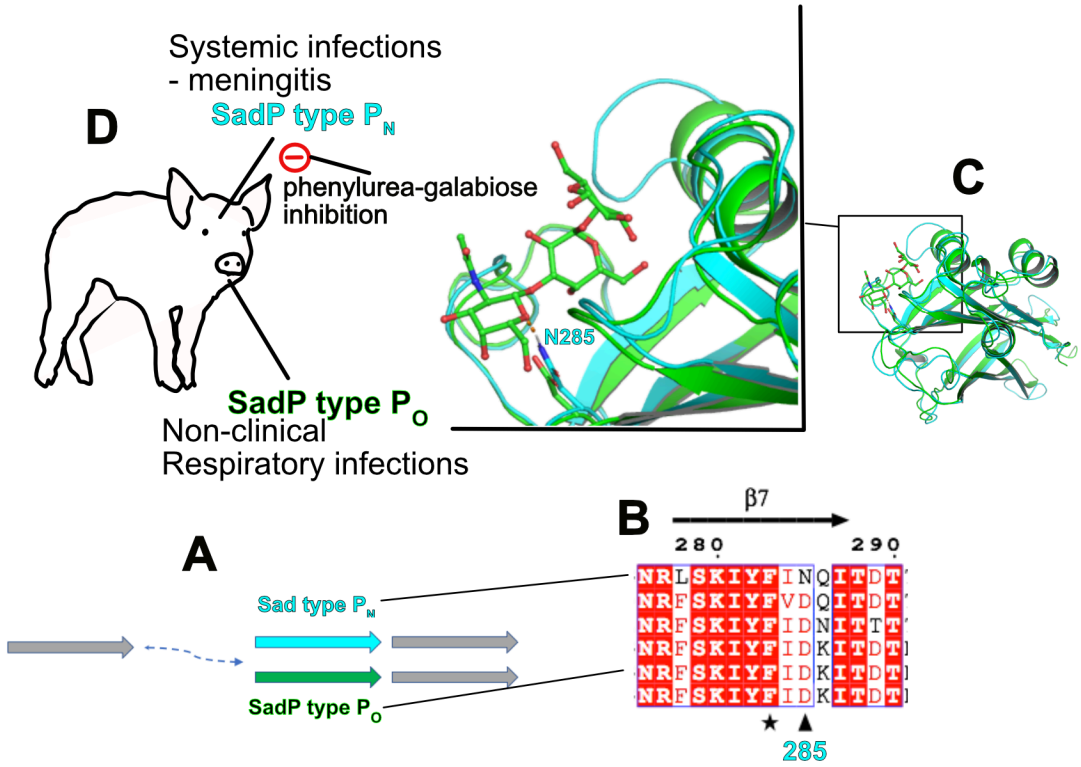

Systemic infections
- meningitis
D
SadP type P_N
phenylurea-galabiose
inhibition
SadP type P_O
Non-clinical
Respiratory infections
N285
C
B
β7
280
290
A
Sad type P_N
SadP type P_O
285

Figure legends

Fig 1. Homology of SadP types P_N and P_O galabiose-binding domains and their distribution in systemic, respiratory and non-clinical *S. suis* isolates. A. Multiple alignment of SadP galabiose binding domains of type P_N and type P_O strains. Conserved amino acids are shown in shaded red rectangles and white letters. Residues with >70% similarity according to their physicochemical properties are framed in white background and shown with red letters. Residues involved in hydrogen-bond formation with galabiose (Galα1–4Gal) are shown with (▲) and those involved in hydrophobic interactions with a star (★). Residues 139-343 of SadP are included as found in the crystal structure (pdb id 5BOB). Secondary structure elements are shown on the top. Figure was created with ESpript [47]. **B. Distribution of SadP type P_N and P_O alleles in *S. suis* clinical and non-clinical isolates.** Maximum-likelihood core genome phylogeny of 374 *S. suis* isolates from Weinert et al. 2015. The phylogeny was run with RAxML using SNP sites. The nucleotide substitution model used was GTR GAMMA. Tips of the tree are coloured according to the SadP allele detected with SRST2. NA means that with the default parameters neither allele was detected. The inner circle shows the phenotype according to Weinert et al. 2015. Grey non-clinical, red respiratory, blue systemic (brain), green systemic (other) and black unknown. The outer circle is coloured according to the SadP allele detected with SRST2 with the same colours as the tree tips. White NA, red P_N and black P_O.

Fig 2. Different glycolipid binding specificities of SadP type P_N from systemic *S. suis* strains and type P_O SadP from respiratory strains towards pig lung glycolipids and purified globo series glycolipids . A. Comparison of the total non-acid glycosphingolipid fractions from porcine lung and erythrocytes. Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived the total non-acid glycosphingolipid fraction from porcine lung by digestion with *Rhodococcus* endoglycoceramidase II. B. Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived the total non-acid glycosphingolipid fraction from porcine erythrocytes by digestion with *Rhodococcus* endoglycoceramidase II. The identification of individual glycosphingolipid-derived oligosaccharides was based on their determined molecular masses and subsequent MS^2 sequencing. The oligosaccharides identified in the chromatograms are: Gb3, Galα1–4Galβ1–4Glc; H5-2, Fucα2–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc; B5, Galα1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc;

Gb4, GalNAcβ1–3Galα1–4Galβ1–4Glc; A6-2, GalNAcα1–3(Fucα2–3)Galβ1–4GlcNAcβ1–3Galβ1–4Glc. **B. SadP binding tri- and tetraglycosylceramides isolated from porcine lung.** Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiograms obtained by binding of SadP (B), followed by autoradiography for 12 h, as described under "Methods". The solvent system used was chloroform/methanol/water (60:35:8, by volume). The lanes were: Lane 1, fraction PL-1 isolated from pig lung, 1 μg; Lane 2, fraction PL-2 isolated from pig lung, 1 μg; Lane 3, fraction PL-3 isolated from pig lung, 1 μg; Lane 4, fraction PL-4 isolated from pig lung, 1 μg; Lane 5, reference globotetraosylceramide (GalNAcβ1–3Galα1–4Galβ1–4Glcβ1Cer), 2 μg; Lane 6, reference globotriaosylceramide (Galα1–4Galβ1–4Glcβ1Cer), 2 μg. **C. TLC overlay assay with purified glycolipids**, **SadP-D282 type P_N** 1. Gb2, galabiaosylceramide 2 μg, 2. iGb3, isoglotriosylceramide 4 μg, 3 Gb3, globotriosylceramide 4 μg , 4. Gb4, globotetraosylceramide 4 μg, 5. Gb5, Forssman 4 μg, 6. P1 pentaosylceramide 4 μg. **D. SadP-6407 type P_O** 1. Lactosylceramide 4 μg, 2. Galabiaosylceramide 4 μg, 3. Globotriaosylceramide 4 μg, 4. Globotetraosylceramide 4 μg, 5. P1, pentaosylceramide 4 μg, 6. Forssman pentaosylceramide 4 μg, 7. Non-acid glycosphingolipids of porcine small intestine (blood group O) 40 μg.

Fig 3. SadP binding to EA.hy926 cell line. A. Binding of S. suis D282 wild type serotype 2 strain and the SadP insertion mutation to EA.hy926 cells grown on coverslips. The binding of the wild type bacteria was inhibited with 10 μg/ml of pigeon ovomucoid. Flow cytometry analysis of SadP(31-328) type P_N binding to EA.hy926 cells. **B.** Buffer control. **C.** SadP adhesin (0.4 μg/ml) and **D.** SadP adhesin in the presence of 10 μg/ml of pigeon ovomucoid.

Fig 4. Oligosaccharide specificity of SadP from systemic (type P_N) and respiratory (type P_O) *S. suis* strains analyzed with glycans representing the terminal saccharide structure of Gb3 and Gb4. A. and B. ITC of SadP type P_N and type P_O with Galα1–4Galβ1– and GalNAcβ1–3Galα1–4Galβ1– TMSEt derivatives **C.** AlphaScreen inhibition assay with Galα1–4Galβ1– and 3'Phenylurea-Galα1–4Galβ1– methoxyphenyl (-PMP = 4-methoxyphenyl). **D**. AlphaScreen inhibition assay of Galα1–4Galβ1– (compound

C10) and 3'Phenylurea-Galα1–4Galβ1– methoxyphenyl (compound C53) with recombinant N-terminal domains cloned from 4 different type P_O *S. suis* strains **E**. Structures of the ligands in this study.

Fig 5. A. Single amino acid site-specific mutation N285D of SadP from systemic strains changes its Gb4 binding specificity to type P_O Gb3 binding specificity of respiratory strains. A. Inhibition of WT SadP(125-329) and its site-specific mutant SadP(125-329)N285D with the dilutions series (1.25 to 320 μM, duplicate determinations) of TMSEt (2-trimethylsilylethyl) glycosides of Galα1–4Gal and GalNAcβ1–3Galα1–4Gal representing the Gb3 and Gb4 oligosaccharides. **B.** Inhibition assay with 5 μM of Galα1–4Gal and GalNAcβ1–3Galα1–4Gal TMSEt glycosides. **C.** ITC measurements of WT SadP and N285D mutant with the TMSEt glycosides of Galα1–4Gal and GalNAcβ1–3Galα1–4Gal representing the Gb3 and Gb4 oligosaccharides.

Fig 6. SadP and $SadP_{N285D}$ binding to liposomes. POPC/glycolipid liposomes (4, 0.4 and 0.04 nmol/dot) and POPC/cholesterol/globosylceramide liposomes (4, 0.4 and 0.04 nmol/dot) were applied onto PVDF membrane. WT, His-tagged SadP(125-329) type P_N; N285D, site-specific mutant of SadP(125-329); Replicate membranes containing dotted liposomes with or without cholesterol (see Methods) were probed with **SadP and $SadP_{N285D}$**. The bound proteins were detected with anti-His and HRP-labelled antibodies as described in the methods and membrane was imaged with FujiLAS-4000. The globo series glycolipids used were: Gb4, d18:1-24:0 + d18:1 – 16:0; Gb3/1, Gb3 d18:1-24:0; Gb3/2, Gb3 d18:1-16:0; Gb3/3, Gb3 d18:1-h16:0.

Fig 7. A. Arrangement of the five site-specifically mutated SadP(125-329)N285D molecules in the crystallographic asymmetric unit. Each molecule is shown as ribbon and colored differently. **B.** Ribbon diagram of SadP(125-329)N285D structure. α-Helices are shown in yellow color and β-strands in cyan. D285 is depicted as sticks. **C.** Structural superposition of Galα1–4Gal -bound Fhc (PDB 5BOA) structure (identical to SadP type P_N (pink) onto $SadP(125\text{-}329)_{N285D}$ (cyan). Residues involved in binding interactions are shown

in stick representation. **D.** STD-NMR of binding of wild type SadP(125-329) type P_N and its site-specific mutant N285D. Peaks in the STD spectra indicate hydrogens (C-H) that are in close proximity to the protein.

Fig 8. Molecular dynamics of SadP type P_N binding to Gb4 saccharide and glycomimetic phenylurea-galabiose inhibitor. Representative MD snapshots (at 90 ns) of SadP. WT in complex with **A**. Galα1–4Gal, **B**. GalNAcβ1–3Galα1–4Galβ, and **C.** C3´-phenylurea-Galα1–4Galβ. Representative MD snapshots of SadP N285D in complex with **D.** Galα1–4Gal, **E.** GalNAcβ1–3Galα1–4Galβ, and **F.** C3´-phenylurea-Galα1–4Galβ.

Fig 9. Summary of the results. A. SadP type P_N (cyan) and type P_O (green) are putative duplicates of the downstream SadP homolog (grey). **B.** Conservative change of type P_N SadP asparagine-285 to aspartic acid of SadP type P_O. **C.** This study has shown the mechanism how N285 contributes to the binding of terminal GalNAcβ1- saccharide of Gb4 by a specific interaction with GalNAc hexose ring oxygen (orange dashed line). The 3D-structure of SadP type P_O from type 4 *S. suis* 6407 was obtained by homology modelling (Swiss Prot) and superimposed structures of type P_N (cyan) in complex with GalNAcβ1–Galα1–4Gal and type P_O (green) were created with Pymol. The structures suggest that the abolishment of Gb4 binding is based on the same mechanism as shown with N285D mutant. **D.** The results of the binding mechanisms of SadP type P_N with Gb4 and the distribution of SadP subtypes in *S. suis* clinical and non-clinical strains suggest that SadP Gb4 binding is associated with strains causing systemic disease. Therefore, the molecular mechanism of SadP type P_N binding to Gb4 could be targeted to prevent *S. suis* systemic diseases.

Supporting information

S1 Fig. LC-ESI/MS of the native fraction PL-1 from pig lung. (A) Base peak chromatogram from LC-ESI/MS of fraction PL-2 from pig lung. (B) MS^2 of the ion at *m/z* 1132 (retention time 14.1 min). The interpretation formula shows the deduced glycosphingolipid structure.

S2 Fig. LC-ESI/MS of the native fraction PL-2 from pig lung. (A) Base peak chromatogram from LC-ESI/MS of fraction PL-2 from pig lung. (B) Mass chromatogram of *m/z* 1022. (C) Mass chromatogram of *m/z* 1132. (D) MS^2 of the ion at *m/z* 1022 (retention time 13.1 min). (E) MS^2 of the ion at *m/z* 1132 (retention time 12.5 min). (F) Interpretation formulas showing the deduced glycosphingolipid structures.

S3 Fig. LC-ESI/MS of the oligosaccharides obtained by digestion of fraction PL-1, PL-2, reference globotriaosylceramide, and reference isoglobotriaosylceramide with *Rhodococcus* endoglycoceramidase II. A. Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction PL-1. B. Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction PL-2. C. Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from reference globotriaosylceramide (Galα1–4Galβ1–4Glcβ1–O–Cer). D. Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from reference isoglobotriaosylceramide (Galα1–3Galβ1–4Glcβ1–O–Cer). E. MS^2 of the ion at *m/z* 503 (retention time 18.4 min) in (A). F. MS^2 of the ion at *m/z* 503 (retention time 17.7 min) in (B). G. MS^2 of the ion at *m/z* 503 (retention time 17.4 min) in (C). H. MS^2 of the ion at *m/z* 503 (retention time 20.2 min) in (D). The interpretation formula shows the deduced oligosaccharide structure. The identification of oligosaccharides was based on their retention times, determined molecular masses and subsequent MS^2 sequencing.

S4 Fig. LC-ESI/MS of the oligosaccharides obtained by digestion of fraction PL-4, and reference globotetraosylceramide with *Rhodococcus* endoglycoceramidase II. A. Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction PL-4. B. MS^2 of the ion at *m/z* 706 (retention time 18.4 min) in (A). C. MS^2 of the ion at *m/z* 706 (retention time 18.6 min) from LC-ESI/MS of the oligosaccharides

derived from reference globotetraosylceramide (GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–O–Cer). D. MS^2 of the ion at *m/z* 998 (retention time 19.4 min) in (A). E. MS^2 of the ion at *m/z* 998 (retention time 21.4 min) in (A). The interpretation formula shows the deduced oligosaccharide structure. The identification of oligosaccharides was based on their retention times, determined molecular masses and subsequent MS^2 sequencing.

S5 Fig. SadP types P_N and P_O glycolipid binding specificity analysed with TLC overlay analysis and microwell binding assay: A. Decreasing glycolipid dilutions from 2 to 0.2 µg per lane probed with SadP type P_N full length and it's N-terminal domain SadP(31-328). **B.** Microwell binding assay with radiolabelled SadP type P_N to 250 ng of glycolipids, Gb2 galabiosylceramide, Gb2-S Galα1–4Galβ1–O-*bis*-$(SO_2\text{-}C_{16}H_{33})_2$, Gb3 non-OH globotriaosylceramide with non-hydroxy ceramide, Gb3 OH globotriaosylceramide with hydroxy ceramide, iGb3 isoglobotriaosylceramide, Gb4, globotetraosylceramide, P1 pentaosylceramide. Leb Lewis b hexaosylceramide.

S6 Fig. SDS-PAGE of the type P_N WT SadP(125-329) and the site-specific mutants N285D and E292Q, deletion Δ244-246 and N285D&Δ244-246 mutants were constructed into the type P_N SadP(125-328) cloned into the plasmid pET28a. In addition, the 28 amino acid long region H216-D246 of the type P_N was replaced by the corresponding region of type P_O.

S7 Fig. A. Binding of SadP wild type and site-specific mutants to Galα1–4Gal analysed with AlphaScreen**. B - F.** Inhibition assay for the binding of site-specific mutants of PSAD1-5 (PSAD-1 Δ244-246, PSAD-2 N285D, PSAD-3 E292Q, PSAD-4 N285D&Δ244-246 and PSAD-5 H216-D246delinsDELFNRFPNKVDSTNNGDGAPFRFFNKE (replacement of aa 216-242 into corresponding sequence of type P_O of strain 6107).

S8 Fig. Structure analysis of TMSEt (2-trimethylsilylethyl) glycosides

S9 movie. MD simulation of type P_N WT SadP(125-329) and Galα1–4Gal

S10 movie. MD simulation of N285D SadP(125-329) and Galα1–4Gal

S11 movie. MD simulation of of type P_N WT SadP(125-329) and GalNAcβ1–3Galα1–4Gal

S12 movie. MD simulation of of type P_N WT SadP(125-329) and 3'-phenylurea- Galα1–4Gal

S13 movie. MD simulation of N285D SadP(125-329) and GalNAcβ1–3Galα1–4Gal

S14 movie. MD simulation of N285D SadP(125-329) and 3'-phenylurea- Galα1–4Gal