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The ADP-glucose pyrophosphorylase from *Streptococcus mutans* provides evidence for the regulation of polysaccharide biosynthesis in Firmicutes

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Summary

Streptococcus mutans **is the leading cause of dental caries worldwide. The bacterium accumulates a glycogen-like internal polysaccharide, which mainly contributes to its carionegic capacity. *S. mutans* has two genes (*glgC* and *glgD*) respectively encoding putative ADP-glucose pyrophosphorylases (ADP-Glc PPase), a key enzyme for glycogen synthesis in most bacteria. Herein, we report the molecular cloning and recombinant expression of both genes (separately or together) followed by the characterization of the respective enzymes. When expressed individually GlgC had ADP-Glc PPase activity, whereas GlgD was inactive. Interestingly, the coexpressed GlgC/GlgD protein was one order of magnitude more active than GlgC alone. Kinetic characterization of GlgC and GlgC/GlgD pointed out remarkable differences between them. Fructose-1,6-bis-phosphate activated GlgC by twofold, but had no effect on GlgC/GlgD. Conversely, phospho-*enol*-pyruvate and inorganic salts inhibited GlgC/GlgD without affecting GlgC. However, in the presence of fructose-1,6-bis-phosphate GlgC acquired a GlgC/GlgD-like behaviour, becoming sensitive to the stated inhibitors. Results indicate that *S. mutans* ADP-Glc PPase is an allosteric regulatory enzyme exhibiting sensitivity to modulation by key intermediates of carbohydrates metabolism in the cell. The particular regulatory properties of the *S. mutans* enzyme agree with phylogenetic analysis, where GlgC and GlgD proteins found in other Firmicutes arrange in distinctive clusters.**

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Introduction

Macromolecules consisting of α-1,4 linked glucose residues with varying amounts of α-1,6 branches are ubiquitous in living organisms, in which they provide short- or long-term storage for readily available carbon (Ballicora *et al.*, 2003; 2004). For example, glycogen as well as other similar α-1,4 glucans have been reported in more than 50 different bacterial species. The polysaccharide is not restricted to any class of prokaryotes as many Gram-negative and Gram-positive bacteria as well as archaebacteria have been reported to accumulate glycogen (Preiss, 2009). Genetic and biochemical features of glycogen synthesis have been well studied in *Escherichia coli* and many other Gram-negative bacteria (Ballicora *et al.*, 2003; 2004). Conversely, reports on the characterization of the enzymes involved in glycogen metabolism in Gram-positive bacteria are scarce, which represents an important deficit for the understanding of carbon metabolism in these organisms (Ballicora *et al.*, 2003; Preiss, 2009). Bacterial glycogen synthesis occurs through ADP-glucose (ADP-Glc) serving as the glucosyl donor for elongation of the α-1,4-glycosidic chain catalysed by glycogen synthase (GSase, EC 2.4.1.21). In such a route, ADP-Glc synthesis is mediated by ADP-Glc pyrophosphorylase (ADP-Glc PPase, EC: 2.7.7.27); this reaction being the key regulatory step in the pathway (Ballicora *et al.*, 2003). Although some experiments suggested that glycogen plays a role in the survival of bacteria, its precise function remains unclear (Preiss, 2009).

Prokaryotic ADP-Glc PPases have been grouped in eight different classes on the basis of their allosteric regulatory and structural properties and in comparison with metabolic characteristics of the respective microorganism (Ballicora *et al.*, 2003; 2004). In all bacteria so far characterized only one gene (*glgC*) was identified as coding for ADP-Glc PPase, but in *Bacillus subtilis* (Kiel *et al.*, 1994)

and in *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) (Takata *et al*., 1997) two genes (*glgC* and *glgD*) were found. In fact, the ADP-Glc PPase from *G. stearothermophilus* was characterized by recombinant expression of both genes to determine that it arranges in a heterotetrameric active form that is insensitive to regulation; this in clear difference with the homotetrameric structure and regulatory characteristics found in other bacteria (Ballicora *et al*., 2003; 2004). An exhaustive analysis performed using the information derived from genomes from prokaryotes elucidated in the last years show that the occurrence of the two genes coding for ADP-Glc PPase is common to all Gram-positive bacteria grouped as Firmicutes (low G+C content). This called our attention about the scarce information concerning the properties of enzymes involved in the glycogen biosynthetic pathway in this important group of bacteria.

Oral streptococci are sugar-fermentative Gram-positive (low G+C content) bacteria comprising at least 19 distinct species that are a significant proportion in normal microbiota of the upper respiratory tract. In these microorganisms, the synthesis and later degradation of glycogen (or internal polysaccharide, IPS, a glycogen-like macromolecule) has been shown to be an important factor in the development of dental caries (Harris *et al*., 1992; Spatafora *et al*., 1995; 1999). Specifically, when exogenous carbohydrates are exhausted, these organisms use the IPS to produce and excrete acids, reducing the pH in the environment (Huis in 't Veld and Backer Dirks, 1978; Preiss, 1984). The latter biochemical behaviour is distinctive of streptococci when compared with metabolic characteristics of other oral bacteria that are unable to synthesize IPS. The acid formed from polysaccharide catabolism may be of significance in the development of dental caries (Harris *et al*., 1992; Spatafora *et al*., 1995; 1999; Busuioc *et al*., 2009). Recently, it has been shown that inactivation of the *glgA* gene (SMU1536), encoding a putative GSase, prevents accumulation of IPS in *S. mutans* UA159 (Busuioc *et al*., 2009). Thus, it is important to achieve a complete characterization of the pathway leading to IPS synthesis and its potential metabolic regulation.

In this work we report the molecular cloning of two genes (*glgC* and *glgD*) encoding for ADP-Glc PPase in *S. mutans*. The heterologous expression of the cloned genes served to produce and characterize GlgC, GlgD, and GlgC/GlgD forms of recombinant proteins. Results are discussed in relation with possible physiological regulation of glycogen synthesis in *S. mutans* through the allosteric modulation of the activity of ADP-Glc PPase by key metabolites of the carbon and energy pathways in the bacterium. Also analysed is the relevance that such regulatory mechanisms could have for determining virulence and cariogenicity in *S. mutans*.

Results

Isolation and analysis of the genes coding for ADP-Glc PPase in S. mutans

The genome elucidated for *S. mutans* UA159 has two genes, *glgC* (SMU 1538) and *glgD* (SMU 1537) putatively coding for ADP-Glc PPase. Both genes belong to a *glgBCDA* operon, where *glgA* and *glgB* code for putative glycogen synthase and glycogen branching enzyme respectively (Ajdic *et al*., 2002). Thus, the operon is related with a pathway leading to glycogen synthesis (Ballicora *et al*., 2003), which has been shown to be active in *S. mutans* (Kim *et al*., 2008; Busuioc *et al*., 2009); although the respective proteins have not been biochemically characterized. The latter prompted us to search for determining the functional role of *glgC* and *glgD* in *S. mutans*, because of the known regulatory function of ADP-Glc PPase in the pathway for glycogen synthesis in bacteria (Ballicora *et al*., 2003; 2004). Although it could be possible to amplify both genes (*glgC* and *glgD*) together [as it was reported for the heterotetrameric ADP-Glc PPase from *G. stearothermophilus* (Takata *et al*., 1997)], we decided to separately amplify each single gene from *S. mutans* ATCC 25175, using specific primers properly designed based on the database information available for *S. mutans* UA159 (Ajdic *et al*., 2002). Both complete sequences coding for the two subunits of ADP-Glc PPase from *S. mutans* ATCC 25175 have been submitted to the NCBI (GenBank Accession Numbers KC473822 and KC473823 for *glgC* and *glgD* respectively). Amino acid sequence of GlgC from *S. mutans* ATCC 25175 was 100% identical to the respective protein from the fully sequenced *S. mutans* strains, (UA159, NN2025, LJ23 and GS-5); instead, GlgD from the ATCC 25175 strain showed three substitutions (Asp^{143} by Asn, Ala^{149} replacing Val and Tyr^{354} by Ile) regarding the reference *S. mutans* UA159 strain. This seems to be well conserved genes in the species since both GlgC and GlgD proteins are present in the 88 *S. mutans* isolated retrieved in the NCBI database.

The *S. mutans* GlgC and GlgD have 25.5% identity between them, which is very similar to the identities regarding *G. stearothermophilus* GlgC and GlgD proteins (28.2%). The *S. mutans glgC* gene codes for a 42.4 kDa protein, which is 36.8% and 33.9% identical to GlgC from *E. coli* and *Agrobacterium tumefaciens* respectively; both ADP-Glc PPases that have been extensively characterized (Ballicora *et al*., 2003; 2007; Bejar *et al*., 2004; 2006) and even crystallized for the latter case (Cupp-Vickery *et al*., 2008). In addition, *S. mutans* GlgC is 51.9% and 29.6% identical at the amino acid level to GlgC from *G. stearothermophilus* and *Streptomyces coelicolor*, respectively, which are the only two Gram-positive bacteria where ADP-Glc PPase was so far characterized (Takata *et al*., 1997; Asencion Diez *et al*., 2012). The *S. mutans*

GlgD subunit is a 42.2 kDa protein that shares a 27.1% identity with the GlgD from *G. stearothermophilus* and only 13.8% with the large subunit of the enzyme from *Solanum tuberosum*. The occurrence of two genes coding for respective proteins related to ADP-Glc PPase found in Firmicutes is similar with what is found in green algae and higher plants. In prokaryotes the genes code for polypeptides α and δ, whereas in eukaryotes they code for subunits α and β. It is worth to point out that, although homologous, proteins δ (GlgD) and β (plant large subunit) exhibit important structural differences (Takata *et al*., 1997; Ballicora *et al*., 2003; 2004; Preiss, 2009).

Expression and purification of recombinant GlgC, GlgD and GlgC/GlcD proteins

We subcloned the *S. mutans glgC* and *glgD* genes into expression vectors allowing the production of the polypeptides GlgC and GlgD separately or the coexpression of both to produce the heteromeric GlgC/GlgD protein. We primarily used the compatible plasmids pMAB5 and pMAB6 to construct [pMAB6/*glgC*] and [pMAB5/*glgD*] expression vectors useful to transform *E. coli* AC7OR1-504 cells, a system that has been functional to study the heteromeric ADP-Glc PPase from potato tuber (Iglesias *et al*., 1993). Cells expressing *S. mutans* GlgC, GlgD or GlgC/GlgD proteins were harvested, resuspended in *buffer A* and after disruption soluble fractions were analysed for ADP-Glc PPase activity (radiometric method) and for protein expression by Western blots (Fig. 1). As shown in Fig. 1A, ADP-Glc PPase specific activity in crude extracts from non-transformed *E. coli* AC70R1-504 cultures (basal activity) was negligible (0.003 ± 0.001 mU mg^{-1}), a value that increased by more than two or three orders of magnitude (note logarithmic scale for enzyme activity in Fig. 1A) in samples obtained from cells expressing *S. mutans* GlgC or GlgC/GlgD respectively. Distinctively, extracts from cells transformed with [pMAB5/*glgD*] alone did not exhibit a specific activity (0.005 ± 0.001 mU mg^{-1}) significantly different than the basal value found for non-transformed cells (Fig. 1A), which may suggest that GlgD would be physiologically negligible (see below).

To confirm the presence of GlgC, GlgD or GlgC/GlgD in the respective crude extract, immunoassays were performed with corresponding samples antibodies raised against *G. stearothermophilus* GlgC and GlgD proteins. As shown in Fig. 1B, anti-GlgC serum recognized a 45 kDa band in extracts from cells expressing *S. mutans* GlgC and GlgC/GlgD. Instead, there was no detection either in the sample expressing GlgD or in the non-transformed *E. coli* AC70R1-504 crude extract. A 45 kDa band was also recognized in samples from cells expressing GlgC/GlgD or GlgD when anti-GlgD serum was used (Fig. 1C), although no recognition was observed in the lane corresponding to

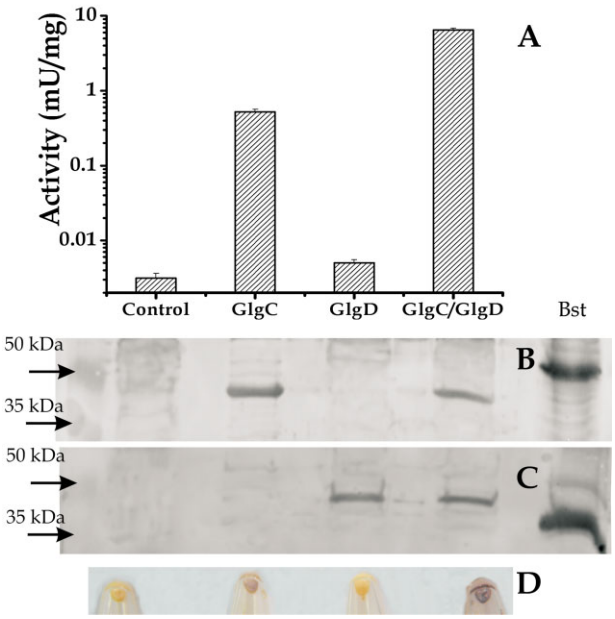

Fig. 1. Expression of *S. mutans* ADP-Glc PPase in *E. coli* AC70504-RI strain. Histogram (A) shows specific ADP-Glc PPase activitiy in soluble fractions from non-transformed *E. coli* AC70R1-504 cultures (Control), or cells transformed to express GlgC, GlgD or GlgC/GlgD from *S. mutans* (top side). Bars indicate standard deviation of activity measurements. The bottom corresponds to immunodetection of the respective soluble fraction from *E. coli* AC70R1-504 cells with antibodies raised against GlgC (B) or GlgD (C) from *G. stearothermophilus*, as stated under *Experimental procedures*. Lane Control: non-transformed *E. coli* AC70R1-504 cells; Lane GlgC: *E. coli* AC70R1-504 [pMAB6/*glgC*] cells; Lane GlgD: *E. coli* AC70R1-504 [pMAB5/*glgD*] cells; Lane GlgC/GlgD: *E. coli* AC70R1-504 [pMAB6/*glgC*] + [pMAB5/*glgD*]; Lane Gst: recombinant ADP-Glc PPase from *G. stearothermophilus*. Note that GlgC and GlgD from *G. stearothermophilus* are proteins of 50 kDa and 38 kDa respectively (Takata *et al*., 1997). Iodine staining (D) of pellets cells from *E. coli* AC70RI-504 (lacking ADP-Glc PPase activity; Control) after GlgC, GlgD or GlgC/GlgD proteins were expressed (see Experimental procedures).

GlgC alone. Taken together, these results indicate that the most active conformation for *S. mutans* ADP-Glc PPase would arrange a GlgC/GlgD structure, followed by the GlgC protein with 10-fold lower activity. In cultures expressing only GlgD the activity was negligible; thus suggesting that this protein is inactive. Furthermore, the *in vivo* functionality of the recombinant expression of GlgC and/or GlgD subunits was followed by analysing if they reverted the impaired capacity that have *E. coli* AC70R1-504 cells to accumulate glycogen (Fig. 1D). When GlgC or GlgC/GlgD (the active forms of *S. mutans* ADP-Glc PPase) were expressed, glycogen accumulation was clearly detected by means of iodine staining; while in control cells or in those where GlgD (the inactive subunit) was expressed alone, the synthesis of the polysaccharide was undetectable (Fig. 1D).

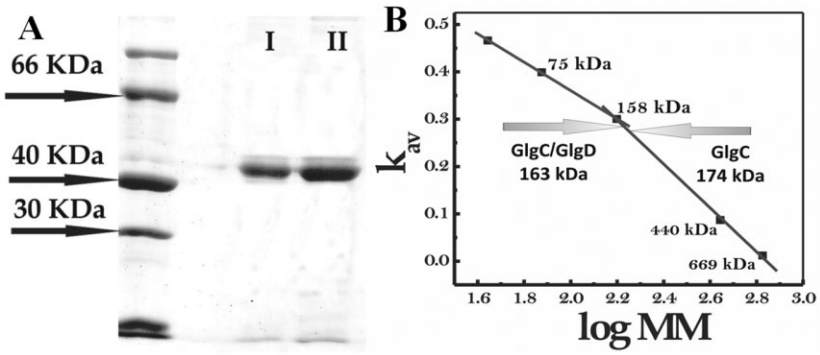

Fig. 2. A. SDS-PAGE of recombinant GlgC and GlgC/GlgD from *S. mutans* after purification. Lane I: purified homotetrameric GlgC. Lane II: purified heterotetrameric GlgC/GlgD. Purifications were conducted as described under *Experimental procedures*. Gels were loaded with 10 to 25 μg of protein per well and stained with Coomassie brilliant blue.
B. Molecular mass (MM) determination, performed by size exclusion chromatography on Superdex 200, as detailed under *Experimental procedures*.

The use of the vector system pRSFDuet-1 was very convenient to reach overexpression of the GlgC, GlgD and GlgC/GlgD proteins from *S. mutans*. In fact, in crude extracts of *E. coli* BL21 (DE3) cells transformed to express GlgC or GlgC/GlgD we measured activities of 0.012 U mg^{-1} or 0.220 U mg^{-1}, respectively, which represent values about 50-fold higher than those shown in Fig. 2 and obtained when using the pMAB system. Also, this strategy facilitated purification of GlgC and GlgC/GlgD by ion exchanged and hydrophobic chromatography, as stated under *Experimental procedures*. The purity and structure of each enzyme form was analysed by SDS-PAGE and size exclusion chromatography (Fig. 2). Both forms of the enzyme were purified to near electrophoretic homogeneity (Fig. 2A). The specific activities reached were 0.56 U mg^{-1} and 6.0 U mg^{-1} for GlgC and GlgC/GlgD respectively. Pure enzymes obtained from pRSFDuet-1 were used for kinetic, regulatory and structural characterization. The quaternary structures of the purified GlgC and GlgC/GlgD proteins were determined and their molecular masses were 174 kDa and 163 kDa respectively (Fig. 2B). These values are in good agreement with theoretical molecular mass (about 176 kDa) calculated for tetrameric forms of each of these enzymes. These results are in concurrence with the quaternary structure of ADP-Glc PPases from different sources previously characterized (Ballicora *et al*., 2003; 2004), including the *G. stearothermophilus* enzyme, which is the only bacterial ADP-Glc PPase coded by two genes that has been studied so far (Takata *et al*., 1997). These results indicate that active forms of ADP-Glc PPase from *S. mutans* are tetrameric in structure, either a homotetramer composed by the active subunit GlgC or a heterotetramer arranged between GlgC and GlgD subunits, the latter giving a form with significantly higher activity.

Kinetic analysis of S. mutans *GlgC and GlgC/GlgD*

Both active forms of *S. mutans* ADP-Glc PPase, GlgC and GlgC/GlgD, were analysed regarding their kinetic and regulatory properties. We first assayed the dependence of the activity with pH. As shown in Fig. S1, GlgC/GlgD had the highest activity at pH 8.0, rapidly decreasing at values lower than pH 7.0 or higher than pH 8.5. GlgC, instead, performed a broader activity profile with pH, exhibiting almost similar activity in the range pH 7.0–9.0. In general, these results are in good agreement with those previously reported elsewhere for ADP-Glc PPases from different sources, exhibiting maximum values of activity at pH ~ 8.0 and very low ones at acidic pH (Ballicora *et al*., 2003; 2004). Dental plaque reaches acidic pH values although it was proposed that *S. mutans* proceeds with different strategies to intracellularly counteract this acidogeny (Lemos *et al*., 2005; Lemos and Burne, 2008). When we performed enzyme assays at pH values between 5 and 6 (using acetate/acetic acid buffer) activity remained around 10% of that measured at pH 8.0 (data not shown), which suggest that under physiological acidogenic periods synthesis of ADP-Glc (and also of glycogen) would be disfavored. On the other hand, it is well known that the divalent metal ion Mg^{2+} is the essential cofactor for ADP-Glc PPase (Ballicora *et al*., 2003; 2004). Both *S. mutans* enzymes (GlgC and GlgC/glgD) exhibited no catalytically activity in the absence of Mg^{2+} and they were fully active at 10 mM of the divalent cation. We also observed that Mn^{2+} could replace Mg^{2+} as a cofactor for GlgC and GlgC/GlgD (data not shown). Table 1 illustrates that both forms of the enzyme exhibited similar affinity for the essential cofactor Mg^{2+}.

Also detailed in Table 1 are the kinetic parameters for the substrates Glc-1P and ATP, which are of value to make a comparative analysis between the properties exhibited by the forms GlgC and GlgC/GlgD of the *S. mutans* ADP-Glc PPase. It is shown that the behaviour of GlgC was slightly sigmoid for ATP with positive cooperativity and depicted a hyperbolic plot for Glc-1P, reaching a V_{max} of 0.62 U mg^{-1}. This form of the enzyme exhibited similar affinity toward ATP and Glc-1P, which for the latter substrate is noticeably low when compared with other ADP-Glc PPases that typically have $S_{0.5}$ values for Glc-1P around 0.1 mM (Ballicora *et al*., 2003; 2004). Even more, the $S_{0.5}$ value for Glc-1P of *S. mutans* GlgC is 20-fold higher regarding the homotetrameric conformation of *G. stearothermophilus* ADP-Glc PPase (Takata *et al*., 1997). Concerning GlgC/GlgD, satu-

Table 1. Kinetic parameters for *S. mutans* GlgC and GlgC/GlgD.

Enzyme	Parameter		
	$S_{0.5}$ (mM)	n_H	V_{max} (U mg^{-1})
GlgC			
ATP	3.42 ± 0.21	1.2	0.62 ± 0.07
Glc-1P	3.44 ± 0.19	0.9	
Mg^{2+}	1.55 ± 0.13	1.7	
GlgC/GlgD			
ATP	1.18 ± 0.12	3.7	6.32 ± 0.37
Glc-1P	0.07 ± 0.01	1.2	
Mg^{2+}	2.36 ± 0.18	1.6	

Assays were carried out as described under *Experimental procedures*. $S_{0.5}$ is the amount of substrate needed to reach 50% of V_{max}; whereas n_H is the Hill number or coefficient. Values of $S_{0.5}$ and n_H were calculated from averaged data from three independent experiments, using regression analysis.

ration curves showed a slight deviation from the hyperbolic behaviour for Glc-1P; while ATP consumption depicted sigmoid plots with positive cooperativity (Table 1). There is a remarkable increase (50-fold) in the apparent affinity of GlgC/GlgD for Glc-1P regarding homotetrameric GlgC (see also Fig. S2). Also, the heterotetrameric enzyme exhibited 10-fold higher V_{max} and twofold lower $S_{0.5}$ for ATP when compared with the kinetic behaviour of GlgC (Table 1). Both forms of the *S. mutans* enzyme exhibited similar affinity toward Mg^{2+} and they performed sigmoidal saturation kinetics for the divalent cation (Table 1).

It has been determined that pyrophosphorylases are relatively specific for their respective substrates, although some exceptions have been reported. For example, dTTP is a poor substrate for UDP-Glc PPase from some bacteria (Weissborn *et al*., 1994; Bosco *et al*., 2009) and for GDP-mannose (GDP-Man) PPase from *Mycobacterium tuberculosis* and *Leptospira interrogans* are promiscuous regarding nucleotide tri-phosphates (NTPs) (Ning and Elbein, 1999; Asencion Diez *et al*., 2010). We explored the possibility for using different NTPs (UTP, ITP, GTP, dTTP) as substrates alternative to ATP by both *S. mutans* GlgC and GlgC/GlgD. It was observed that both active forms of the enzyme were strictly specific for ATP (data not shown). In a whole view, the kinetic behaviour of the *S. mutans* ADP-Glc PPase indicates a high specificity for substrates; whereas differences in properties between GlgC/GlgD and GlgC strongly suggest that the by itself inactive GlgD subunit has a direct regulatory action for the enzyme activity. This situation resembles the modulating role attributed to the large subunit in ADP-Glc PPase from plants (Ballicora *et al*., 2004), rather than to that neutral function found for the GlgD subunit in the *G. stearothermophilus* enzyme (Takata *et al*., 1997), where no significant difference in kinetic behaviour was observed between the homo- and the heterotetrameric conformations.

Regulatory behaviour: both GlgC and GlgC/GlgD are sensitive to effector molecules

In bacteria, the key limiting step in the pathway leading to glycogen synthesis is that generating ADP-Glc (Ballicora *et al*., 2003; Preiss, 2009). ADP-Glc PPases are allosterically regulated by compounds belonging to the principal route for carbon assimilation in the respective organism, and the specific regulation of the *E. coli* enzyme has been recently studied at the molecular level (Figueroa *et al*., 2011). Activation-inhibition assays were performed for both *S. mutans* GlgC and GlgC/GlgD enzyme conformations with compounds known to be important effectors of ADP-Glc PPases characterized from different sources (Leung *et al*., 1986; Ballicora *et al*., 2003). The effect of several inorganic salts over the activity of both GlgC and GlgC/GlgD was also analysed. Results are summarized in Fig. 3 and Table 2.

We found that fructose-1,6-bis-phosphate (Fru-1,6-bisP) increased by about twofold the activity of the *S. mutans* homotetramer GlgC with an $A_{0.5}$ of 0.25 mM, but the heterotetrameric GlgC/GlgD enzyme was insensitive even when assayed up to 20 mM Fru-1,6-bisP (Fig. 3A). On the other hand, phospho-*enol*-pyruvate (PEP) inhibited GlgC/GlgD activity with an $I_{0.5}$ value of 0.08 mM, whereas the metabolite produced no effect on GlgC activity (Fig. 3B). Conversely, inorganic orthophosphate (P_i) inhibited both *S. mutans* GlgC and GlgC/GlgD enzymes, with $I_{0.5}$ values of 1.35 ± 0.08 mM and 1.00 ± 0.10 mM respectively (Fig. 3C). Notice that although GlgC and GlgC/GlgD enzymes have similar $I_{0.5}$ values for P_i, they have slight differences concerning inhibition behaviour. Particularly, low P_i concentrations (until 0.2 mM) increased by ~ 1.6-fold GlgC/GlgD activity; afterwards the enzyme was inhibited at higher P_i concentrations; instead, GlgC was inhibited in a P_i-concentration dependent manner in the whole range (0–20 mM), as shown in Fig. 4C. On the other hand, glucose-6P, fructose-6P, nor mannose-6P had any effect on the activity of GlgC or GlgC/GlgD. As well, compounds that activate ADP-Glc PPase from different sources (such as pyruvate, 3-phosphoglycerate and ribose-5P) were ineffective to regulate the *S. mutans* enzyme.

We also found significant differences regarding *S. mutans* GlgC and GlgC/GlgD behaviours when the effect of several inorganic salts was analysed (Table 2). As shown, $(NH_4)_2SO_4$ inhibited both GlgC and GlgC/GlgD; the latter being 4.4-fold more sensitive than the former to the salt concentrations. Inhibition of both forms of the enzyme by $(NH_4)_2SO_4$ occurred with sigmoid pattern (data not shown). In addition, NaCl, KCl and NH_4Cl inhibited GlgC/GlgD activity, but not affected GlgC activity, even at salt concentrations as high as 500 mM (see parameters in Table 2). As a whole, $(NH_4)_2SO_4$ was the strongest salt

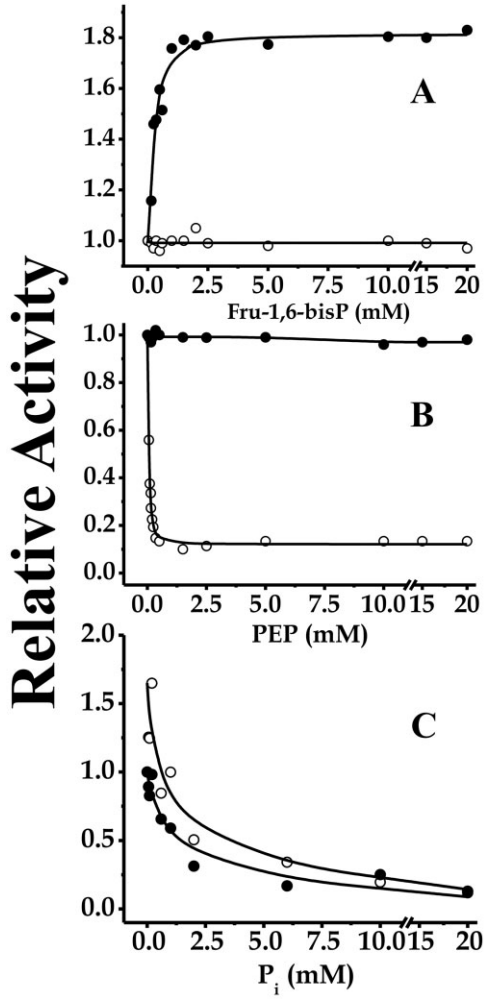

Fig. 3. *S. mutans* GlgC and GlgC/GlgD sensitivity to different metabolites. GlgC is activated by Fru-1,6-bisP (A) although GlgC/GlgD not; instead GlgC/GlgD is inhibited by PEP but not GlgC (B). Both GlgC and GlgC/GlgD are inhibited by P_i (C). Curves represented with empty circles belong to GlgC/GlgD, while those in filled circles to GlgC. Values of relative activity are established as the ratio between the respective activity measured in each condition and that determined in the absence of effector: 0.56 U mg^{-1} (for GlgC) and 6.0 U mg^{-1} (for GlgC /GlgD). Fitting was performed with the Levenberg–Marquardt non-linear least-squares algorithm provided by the computer program Origin™. Hill plots were used to calculate the kinetic constants, which are the mean of at least three independent sets of data reproducible within ± 10%.

producing inhibition, with other salts accomplishing lesser, though significant, inhibition of the *S. mutans* GlgC/GlgD activity. Data suggest that the inhibitory effect is not due to ionic strength but to inhibition exerted by chloride and sulphate anions; which is further supported by the fact that sodium glutamate up to 500 mM exhibited neither effect on the GlgC activity nor GlgC/GlgD (data not shown). In the case of sulphate, the anion was found to be inhibitor of ADP-Glc PPase from plants, mimicking the effect of its structural analog P_i (Jin *et al.*, 2005).

Taking together, these results show that both conformations of the *S. mutans* ADP-Glc PPase are sensitive to different metabolites, which establishes a strong difference regarding regulatory properties among the enzyme from *S. mutans* characterized in this work and that from *G. stearothermophilus* which has been found insensitive to regulation (Takata *et al.*, 1997). Accordingly, results summarized in Table 2 are another remark about the importance that inactive GlgD subunit possess in regulating the behaviour of *S. mutans* ADP-Glc PPase, since different enzyme conformations have particular regulatory properties with diverse sensitivity to allosteric effectors.

The Fru-1,6-bisP effect and the cross-talk between allosteric effectors

After the twofold activation exerted by Fru-1,6-bisP on homotetrameric *S. mutans* GlgC we analysed if the activator produced any effect on the affinity of the enzyme by substrates. Thus, saturation curves for Glc-1P and ATP were conducted at 1.5 mM Fru-1,6-bisP. Table 3 shows that the allosteric effector not only modified V_{max} but also increased the apparent affinity of the enzyme for the substrates ATP and Glc-1P. When similar studies were conducted with the heterotetrameric enzyme no change in substrates affinity was observed (data not shown), indicating that the *S. mutans* GlgC/GlgD ADP-Glc PPase is completely insensitive to this glycolytic intermediate. Results suggest that Fru-1,6-bisP would be a metabolite that activates the homotetrameric conformation of the *S. mutans* ADP-Glc PPase, not only increasing the enzyme activity but also the affinity for substrates, with a main effect in ATP

Table 2. Compounds altering the activity of GlgC and GlgC/GlgD from *S. mutans*.

Effector	GlgC	GlgC/GlgD
Fru-1,6-bisP	Activates $A_{0.5}$ 0.25 ± 0.02 mM 2-fold V_{max} increasing	No effect
PEP	No effect (up to 10 mM)	Inhibits $I_{0.5}$ 0.08 ± 0.01 mM
Pi	Inhibits $I_{0.5}$ 1.35 ± 0.12 mM	Inhibits $I_{0.5}$ 1.00 ± 0.09 mM
$(NH_4)_2SO_4$	Inhibits $I_{0.5}$ 35 ± 2 mM	Inhibits $I_{0.5}$ 8.00 ± 0.73 mM
NaCl	No effect (up to 500 mM)	Inhibits $I_{0.5}$ 45 ± 3 mM
KCl	No effect (up to 500 mM)	Inhibits $I_{0.5}$ 35 ± 3 mM
NH_4Cl	No effect (up to 400 mM)	Inhibits $I_{0.5}$ 40 ± 4 mM

$A_{0.5}$ is the amount of activator needed to reach the 50% of maximal activation, as well $I_{0.5}$ is the inhibitor concentration that reduces the activity by half.

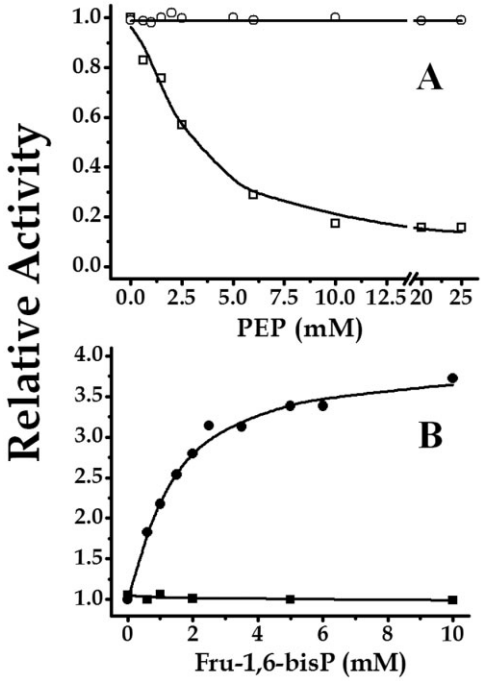

Fig. 4. Combined effects of PEP and Fru-1,6-bisP on the regulation of *S. mutans* ADP-Glc PPase.
A. Response of GlgC. Activity was assayed in the absence (circles) or in the presence (squares) of 1.5 mM Fru-1,6-bisP. In these conditions, now PEP inhibited homotetameric GlgC. The value of 1.0 for the relative activity corresponds to 0.56 U mg^{-1} or 1.2 U mg^{-1} for assays performed in absence or in presence of Fru-1,6-bisP respectively.
B. Response of GlgC/GlgD. Assays were performed in the absence (circles) or in the presence (squares) of 1 mM PEP. Now Fru-1,6-bisP activates the heterotetrameric *S. mutans* ADP-Glc PPase The value of 1.0 for the relative activity refers to 6.0 U mg^{-1} or 1.50 U mg^{-1} for assays performed in the absence or in the presence of PEP respectively. Fitting was performed with the Levenberg–Marquardt non-linear least-squares algorithm provided by the computer program Origin™. Hill plots were used to calculate the kinetic constants, which are the mean of at least three independent sets of data reproducible within ± 10%.

utilization. Albeit, Fru-1,6-bisP activation in homotetrameric GlgC remains insufficient to bring this protein to the same level of activity exhibited by the heterotetramer GlgC/GlgD, which is one order of magnitude higher.

Afterward, we analysed GlgC response to several metabolites when the enzyme was activated by Fru-1,6-bisP. Interestingly, under this condition homotetrameric *S. mutans* GlgC acquired a 'GlgC/GlgD like' behaviour. For example, activated GlgC became sensitive to PEP inhibition, with an $I_{0.5}$ value of 2.55 mM (Fig. 4A). Although this value represents a sensitivity 32-fold lower than that exhibited by GlgC/GlgD to PEP inhibition, it is remarkable that the inhibitory effect on GlgC was triggered by Fru-1,6-bisP. Even more, in the presence of 1.5 mM Fru-1,6-bisP GlgC was also inhibited by NaCl, KCl and NH_4Cl ($I_{0.5}$ values ~ 40–60 mM, data not shown), also resembling the behaviour of GlgC/GlgD. Again, this GlgC salt inhibition conditioned to the presence of Fru-1,6-bisP (1.5 mM) resembles heterotetrameric *S. mutans* ADP-Glc PPase.

The interaction between the effects of Fru-1,6-bisP (activator) and PEP (inhibitor) on GlgC opened the question if something similar could happen for the heterotetrameric form of the enzyme and also include P_i, which was another key metabolite exerting inhibition of the enzyme activity. We analysed if *S. mutans* GlgC/GlgD inhibited by 1 mM PEP to 30% remnant activity was sensitive to different metabolites known to be effectors of ADP-Glc PPase from different sources. From this screening, it was found that Fru-1,6-bisP had an effect, activating the remnant activity of the inhibited heterotetramer by up to ~ 4-fold, thus completely reverting inhibition produced by PEP (Fig. 4B). This activation depicted with an $A_{0.5}$ value of 1.55 mM. A similar behaviour of Fru-1,6-bisP was measured respect interplay with P_i, as the former was able to abolish the inhibition exerted by the latter main metabolite. As a consequence, Fig. 5 shows that a wide range of activity could be determined for GlgC/GlgD depending on relative levels of Fru-1,6-bisP, PEP, and P_i in the assay medium. This interaction between Fru-1,6-bisP, PEP, and P_i in modulating the activity of both, GlgC and GlgC/GlgD would be of physiological relevance, as three major metabolic intermediates (highly related with carbon and energy levels in the bacterial cell) could act as key regulatory metabolites on *S. mutans* ADP-Glc PPase and consequently affecting IPS synthesis.

The above described kinetic and regulatory properties obtained *in vitro* for recombinant GlgC and GlgC/GlgD have to be analysed with respect to conditions taking place in *S. mutans*. The results suggest that if homotetrameric GlgC is present *in vivo* it will not be fully operative since its $S_{0.5}$ value for Glc-1P (around 3 mM) is more than one order of magnitude higher than the levels of the

Table 3. Kinetic parameters for *S. mutans* homotetrameric GlgC in the presence of 1.5 mM Fru-1,6-bisP.

Parameter	ATP	Glc-1P
$S_{0.5}$ (mM)	0.67 ± 0.03	2.04 ± 0.14
Relative affinity[a] (-fold)	5.1 ± 0.4	1.7 ± 0.15
n_H	1.5 ± 0.1	1.7 ± 0.1
V_{max} (U mg^{-1})	1.25 ± 0.11	
Activation[b] (-fold)	2.1 ± 0.3	

a. Relative affinity is the ratio between the respective $S_{0.5}$ values determined in the absence (see Table 1) over in the presence of 1.5 mM Fru-1,6-bisP.
b. Activation was calculated from the ratio of V_{max} values determined in the presence of 1.5 mM Fru-1,6-bisP over that measured in its absence (see Table 1).
$S_{0.5}$ is the amount of substrate needed to reach 50% of V_{max}.

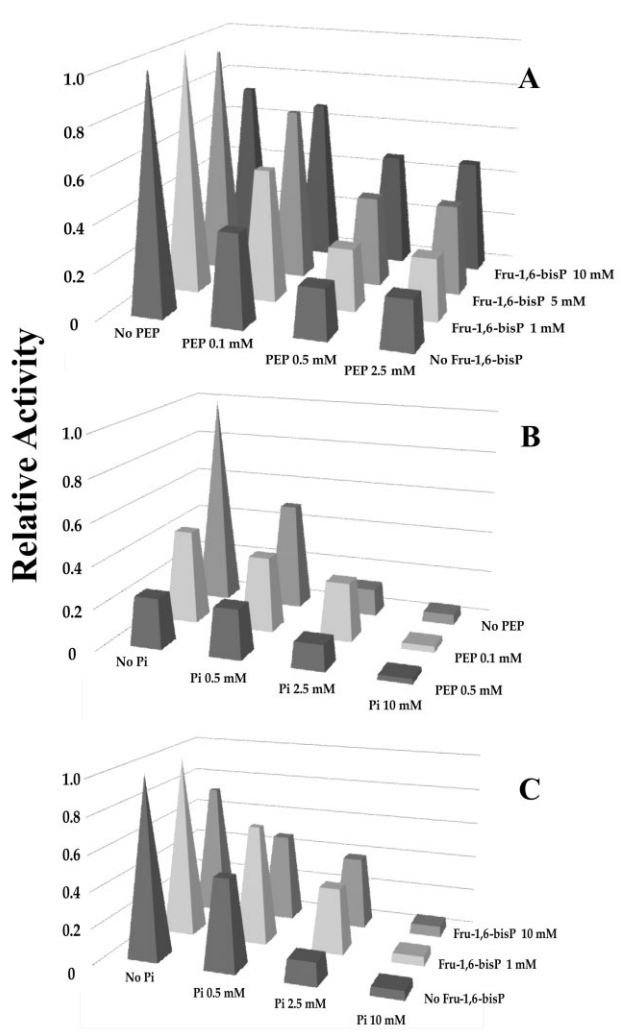

Fig. 5. Interplay between effectors of *S. mutans* GlgC/GlgD. Panel A shows *S. mutans* GlgC/GlgD activity in the presence of different PEP and Fru-1,6-bisP concentrations. Panel B shows P_i inhibition in the absence or the presence of Fru-1,6-bisP 1 mM and 10 mM. Panel C shows the cooperative inhibition effect of PEP and P_i. Reactions were conducted with 1.5 mM ATP and 1 mM Glc-1P. Values of relative activity were calculated as the ratio between the initial velocity measured at a particular condition and that determined in the absence of effector (6.0 U mg^{-1}).

metabolite found in prokaryotic cells (Keevil *et al*., 1984; Takahashi *et al*., 1991; 2010). The biggest effect of GlgD on GlgC was to increase the apparent affinity for Glc-1P by 50-fold, suggesting the heterotetramer as the physiologically operative enzyme. In addition, genes *glgC* and *glgD* seem to be co-transcribed, supporting the idea that the heterotetramer is the effective enzyme form in the bacterium. Enzyme assays in crude extracts, which would have the enzyme in its native quaternary form, were also in agreement with that concept. We obtained crude extracts of *S. mutans* cells grown in micro-aerophilia and minimal medium (with the addition of glucose) and determined that the activity for ADP-Glc synthesis was 19 mU mg^{-1}. Most importantly, ~ 80% of the activity was inhibited by the addition of 1 mM PEP, which is compatible with the properties of GlgC/GlgD (and not GlgC alone). In addition, this was further supported by SDS-PAGE and Western blots of the sample that revealed the presence of polypeptides GlgC and GlgD (Fig. 6).

Structure-to-function relationships and phylogenetic analysis between GlgC and GlgD proteins from Firmicutes

To better understand the particular kinetic behaviour of the *S. mutans* ADP-Glc PPase we made an amino acids alignment (Fig. S3) and we built a homology model (Fig. 7) for each GlgC and GlgD subunits, to identify the occurrence and positioning of domains that are critical for the enzyme functionality. Previous analyses on structure-to-function relationships allowed the identification of key amino acids in the ADP-Glc PPase from different sources (Ballicora *et al*., 2003; 2004; Preiss, 2009). Asp^{142} in the enzyme from *E. coli* was demonstrated to be involved in catalysis, as its mutation to Asn decreased the activity by four orders of magnitude (Frueauf *et al*., 2001). This residue is highly conserved throughout the family of ADP-Glc PPases as well as all over the super-family of sugar–nucleotide pyrophosphorylases (Ballicora *et al*., 2003; 2004). In fact, the homologous residue is present in both the small (Asp^{145}) and the large (Asp^{160}) subunits from the heterotetrameric ADP-Glc PPase from potato tuber (Frueauf *et al*.,

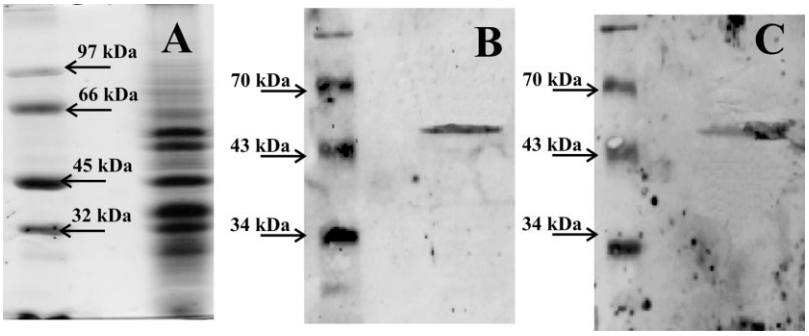

Fig. 6. SDS-PAGE (A) and inmudetection with Anti-C (B) and Anti-D of crude extracts of *S. mutans* ATCC 25175. Cells were growing in a tryptone medium supplemented with vitamins and glucose and harvested after cultured during 16 h.

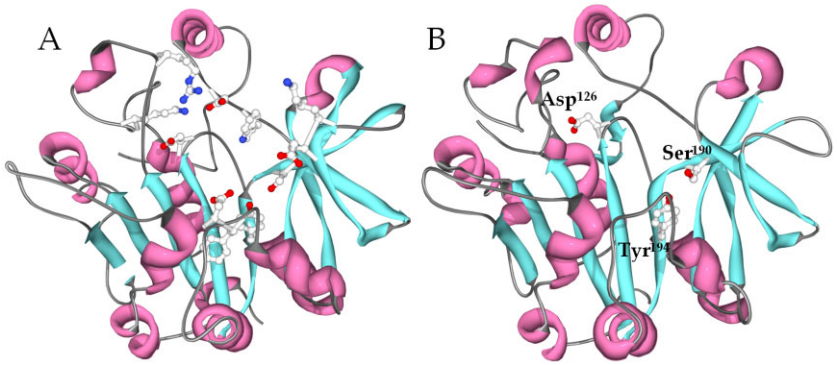

Fig. 7. Modelling of the GlgC (A) and GlgD (B) subunits of the *S. mutans* ADP-Glc PPase. Models were obtained as indicated in *Experimental procedures*. In GlgC subunit, the entire set described for substrates binding and catalysis are conserved (see Preiss, 2009); in modelled GlgD subunit, the conserved amino acidic residues are indicated.

2003). As shown in Fig. S3, this critical Asp residue is also present in both subunits from *S. mutans*, GlgC (Asp^{129}) and GlgD, (Asp^{126}) (see also Fig. 7).

On the other hand, chemical modification (Lee and Preiss, 1986) and site-directed mutagenesis (Hill *et al.*, 1991) studies identified Lys^{195} as an important residue for Glc-1P binding in the *E.coli* ADP-Glc PPase. Lys^{195} makes a salt bridge with the Glc-1P, an interaction that has also been biochemically probed for the homologous residue (Lys^{198}) in the potato tuber enzyme (Fu *et al.*, 1998). This Lys residue is conserved in *S. mutans* GlgC (Lys^{182}) but in the GlgD subunit it is replaced by Asp, a moiety with opposite charge (Fig. S3). Later, Bejar *et al.* (2006) generated a homology model of the three-dimensional structure of the *E. coli* ADP-Glc PPase complexed with ADP-Glc, shedding light on the environment that surrounds the substrates. Thus, Glu^{194}, Ser^{212}, Tyr^{216}, Asp^{239}, Phe^{240}, Trp^{274}, and Asp^{276} were identified close to the glucose moiety of the ADP-Glc ligand. When those amino acids were studied by site-directed mutagenesis, all purified Ala mutants exhibited 1 or 2 orders of magnitude lower apparent affinity for Glc-1P compared with the wild type, confirming their relevant role in substrate interaction (Bejar *et al.*, 2006). All of these amino acid residues are conserved in GlgC (that is, Glu^{191}, Ser^{192}, Tyr^{196}, Asp^{219}, Phe^{220}, Trp^{244} and Asp^{246}) but only the Ser and the Tyr residues are present in *S. mutans* GlgD (Ser^{190} and Tyr^{194} respectively; see Fig. S3 and Fig. 7).

The models shown in Fig. 7 sustain the catatylic properties of the GlgC and GlgD proteins from *S. mutans*. The three-dimensional structure obtained for GlgC (Fig. 7A) depicts the presence of the full set of the above detailed amino acids critical for substrates binding and catalysis (also shown in the alignment in Fig. S3). It is worth mentioning that there is a difference in position 101 (Phe to Tyr) in GlgC, which is near the ATP binding site (Kumar *et al.*, 1988)]. This mutation may justify the relatively high $S_{0.5}$ for ATP exhibited by the former. This explanation is reinforced by: (i) the low affinity of *S. mutans* GlgC for ATP resembles the properties of an *E. coli* Y114F mutant characterized by (Kumar *et al.*, 1988); and (ii) the Tyr residue is not substituted but conserved in the GlgC from the firmicute *G. stearothermophilus* (Fig. S3), which has a behaviour to use ATP similar to the wild type ADP-Glc PPase from *E. coli* (Lee and Preiss, 1986; Kumar *et al.*, 1988; Takata *et al.*, 1997). Concerning the structure modelled for *S. mutans* GlgD (Fig. 7B), it shows a high similitude with ADP-Glc PPase, supporting the idea of a common ancestor for both subunits of bacterial heterotetrameric ADP-Glc PPase (Ballicora *et al.*, 2003; Preiss, 2009). However, the GlgD structure exhibits a disruption of the site for substrates binding and catalysis, as only three (Asp^{126}, Ser^{190} and Tyr^{194}) of the detailed critical amino acids remain conserved (Fig. 7B and Fig. S3). The whole structural characteristics agree with the lack of catalytic functionality found for GlgD.

A main conclusion derived from the kinetic and regulatory properties determined for the homo- and heterotetrameric forms of ADP-Glc PPase from *S. mutans* is that they are markedly different from those previously reported for the enzyme from *G. stearothermophilus* (Takata *et al.*, 1997). Thus, in the latter case it was found that neither GlgD significantly modified the activity of GlgC, nor the homo- or hetero-oligomeric forms of the enzyme exhibited sensitivity to allosteric effectors. Looking for establishing differences in protein structure between the ADP-Glc PPases from *S. mutans* and *G. sterothermophilus* that could explain the dissimilar kinetic and regulatory properties, we made a phylogenetic analysis of GlgC and GlgD proteins from several Firmicutes (Fig. 8 and Table S1). A previous phylogenetic comparison between amino acid sequences of bacterial ADP-Glc PPases allowed differentiate a separate cluster including the enzyme from Gram-positive bacteria with low G+C (Machtey *et al.*, 2012).

Going further in the phylogenetic analysis within the specific cluster of Firmicutes, we found that both proteins, GlgC and GlgD, arrange into branches determining a complex distribution of at least three major clusters grouping Bacillales, Lactobacillales and Clostridiales (Fig. 8). The Clostridiales seems to be a particular group, as it presents many cases where they are found two genes *glgC* or *glgD*, distinguishing three subgroups. The phylo-

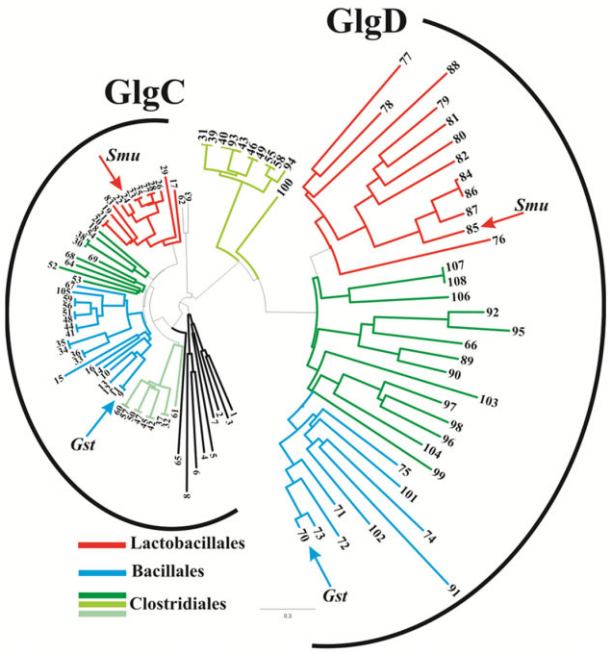

Fig. 8. Phylogenetic tree of GlgC and GlgD from Firmicutes. Sequences of the *glgC* and *glgD* genes were collected and the tree was built as described under *Experimental procedures*. Sequences are numbered with codes indexed in Table S1. Different colours represent different taxonomy: (black) Gram-negative, (blue) Bacillales, (red) Lactobacillales, (light green, green and soft green) different clusters of Clostridiales.

genetic tree shown in Fig. 8 suggests that there was a duplication of one *glgC* gene originating an ancestor that in same organisms duplicate again. Evolution of the duplicated genes would had generated *glgD* and modified *glgC* genes. The subject, principally within the Clostridiales, needs of further studies, not only at the phylogenetic level but mainly in determining kinetic and/or regulatory functions of the different *glgC* and *glgD* genes, which ultimately could be one key parameter to classify them. Besides, Fig. 8 shows that both GlgC and GlgD proteins from *S. mutans* remain included into the group of Lactobacillales in a clear difference from the proteins from *G. stearothermophilus* pertaining to the Bacillales cluster. These results not only support the different kinetic and regulatory properties of ADP-Glc PPase from *S. mutans* compared with that from *G. stearothermophilus*, but also suggest that the enzyme from the Clostridiales may exhibit additional differences in kinetics and regulation.

Discussion

ADP-Glc PPase has been purified from a number of bacteria and the enzyme has been found to be homotetrameric in structure with a subunit molecular size of about 45–50 kDa (Ballicora *et al*., 2003; Preiss, 2009). So far, the only exception is the enzyme from *G. stearothermophilus*, which was characterized as a tetramer composed of two subunits, GlgC and GlgD, and insensitive to allosteric effectors (Takata *et al*., 1997). Concerning quaternary structure, the Firmicutes enzyme shares with higher plants ADP-Glc PPases the property of being heterotetrameric (Takata *et al*., 1997; Ballicora *et al*., 2003), having an additional subunit to the typical GlgC polypeptide (homologous to the plant small or α subunit). However, it is clear that bacterial GlgD (or δ subunit) is quite different with regards to plant ADP-Glc PPases large (or β) subunit not only in structure, but also in functionality (Takata *et al*., 1997; Ballicora *et al*., 2003; 2004; Preiss, 2009). For example, GlgD from *G. stearothermophilus* seems to somewhat increase V_{max} of the GlgC activity and slightly increases GlgC apparent affinity for substrates (Takata *et al*., 1997) while the coexpression of *Arabidopsis* ADP-Glc PPase small subunit (active), APS1, with the different (non-active) large subunits in the organism (APL1, APL2, APL3, and APL4) resulted in heterotetramers with different regulatory and kinetic properties (Crevillen *et al*., 2003; Ballicora *et al*., 2004). Thus, the plasticity provided by large subunits to plant ADP-Glc PPase activity and regulation is a feature absent in GlgD from Firmicutes, the only δ subunit characterized nowadays.

In this work we show the characterization of another heterotetrameric bacterial ADP-Glc PPase belonging to *S. mutans* ATCC 25175. We found two outstanding differences between the latter and the insensitive to regulation enzyme from *G. stearothermophilus*. First, the *S. mutans* ADP-Glc PPase is sensitive to three metabolites acting as activator (Fru-1,6-bisP) and inhibitors (PEP and P_i). Second, a really importance ascribed to the presence of the inactive GlgD subunit in increasing GlgC V_{max}, as well affinity for substrates (remarkably augmenting near 50-fold the affinity towards Glc-1P) and changing sensitivity to allosteric effectors. These differences are in agreement with the phylogenetic study of the proteins GlgC and GlgD from Firmicutes. An analysis combining the kinetic/regulatory and *in silico* results indicate that subunits structurally grouped as Bacillales or Lactobacillales have distinctive functional properties. Additionally, results predict further differences in structure to function relationships for the GlgC and GlgD proteins from the Clostridiales group, which is an open subject for future biochemical studies.

Results obtained for *S. mutans* ADP-Glc PPase also could be analysed concerning the intracellular metabolic scenario (Fig. 9), since there is an important background regarding sugar utilization and IPS synthesis in oral streptococci (Gibbons and Kapsimalis, 1963; Gibbons, 1964; van Houte *et al*., 1969; van Houte and Saxton, 1971; Freedman and Coykendall, 1975; Huis in 't Veld and Backer Dirks, 1978; Birkhed and Tanzer, 1979; Takahashi *et al*., 1991; Spatafora *et al*., 1999; Takahashi and Nyvad,

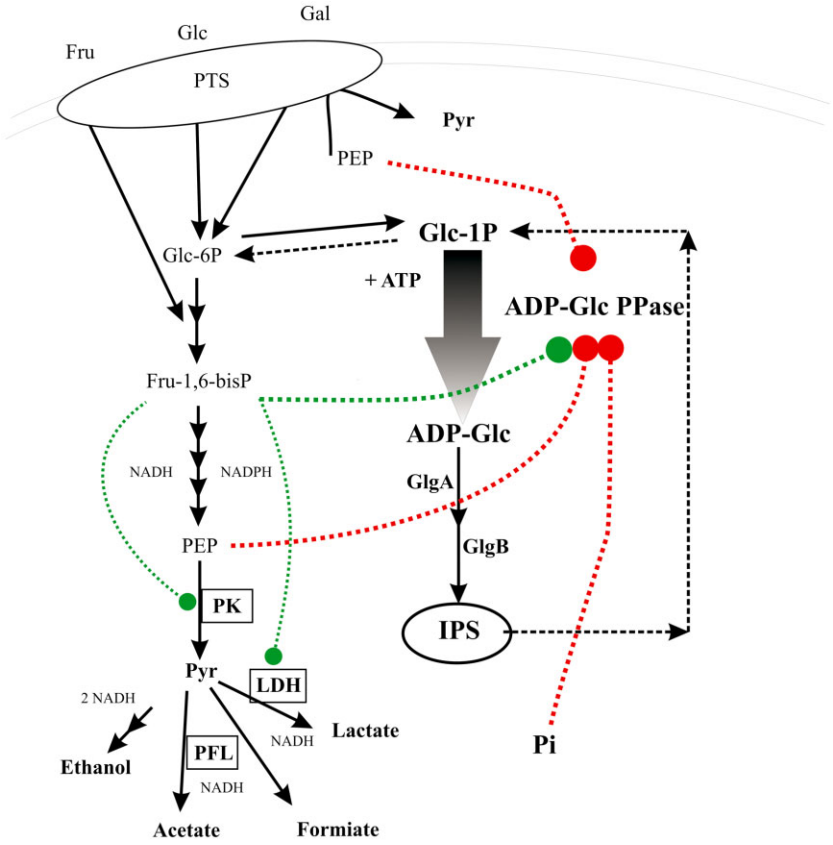

Fig. 9. Metabolic scenario for IPS synthesis linked to acid production in *S. mutans*. ADP-Glc PPase activity is represented in a wide grey arrow. Green and red lines represent the *S. mutans* ADP-Glc PPase allosteric activation or inhibition, respectively, based on the properties determined *in vitro* for GlgC and GlgC/GlgD. Specific abbreviations for the figure. are: Fru, fructose; Gal, galactose; LDH, lactate dehydrogenase; PFL, pyruvate-formiate lyase; P_i, inorganic orthophosphate; PK, pyruvate kinase.

2011). Relationships between Glc consumption and IPS synthesis were established for different *S. mutans* strains (Freedman and Coykendall, 1975) and in *Streptococcus salivarius* (another member of dental plaque), where it has been demonstrated maximal IPS accumulation 4 min after a Glc pulse (Kanapka *et al*., 1971). Nevertheless, enzymatic approach to characterize IPS synthesis (and degradation) was never used, being the present work the first one dealing with kinetic, regulatory and structural properties of enzymes involved in the polysaccharide build-up in *S. mutans* (or any oral bacterium at all). Previously, ADP-Glc PPase and glycogen synthase activities were measured in crude extracts from *S. mutans*, but neither kinetic parameters nor regulatory properties were discussed (Birkhed and Tanzer, 1979). Also, our work clearly establishes that IPS synthesis in *S. mutans* would be regulated at the level of ADP-Glc synthesis, as occurs in most bacteria (Ballicora *et al*., 2003; Preiss, 2009), in contrast to a previous work proposing to Glc-1P formation as the limiting step (Keevil *et al*., 1984).

When sugars are supplied in excess, *S. mutans* can store the overload as IPS (van Houte *et al*., 1969; van Houte and Saxton, 1971; Freedman and Coykendall, 1975; Huis in 't Veld and Backer Dirks, 1978; Birkhed and Tanzer, 1979; Takahashi *et al*., 1991; Spatafora *et al*., 1999; Takahashi and Nyvad, 2011) which is used as a source to acid production when sugar surplus is stopped (van Houte *et al*., 1969; 1970; Tanzer *et al*., 1974; Freedman and Coykendall, 1975; Hamilton, 1977; Spatafora *et al*., 1995; Busuioc *et al*., 2009). In fact, when IPS accumulation is disrupted, *S. mutans* becomes less virulent, less acidogenic and is removed faster from dental plaque (Busuioc *et al*., 2009). Then, carbohydrate utilization and IPS synthesis becomes essential for the survival and persistence of streptococci in plaque environment and the ADP-Glc PPase presented in this work is the link to co-ordinate both features; thus the importance in understanding its kinetic and regulatory behaviours. In addition, *Streptococcus* spp., have a partial tricarboxylic acid cycle (Cvitkovitch *et al*., 1997), lacks an electron transport chain and the oxidative pentose phosphate pathway is absent in pathogenic streptococci (Brown and Wittenberger, 1971; Boyd *et al*., 1995). In this context, sugars fermentation is relevant for generation of energy and reductive power, putting the glycolytic Embden–Meyerhoff–Parnas (EMP) pathway in a prominent role (Skinner and Naylor, 1972; Takahashi *et al*., 1991; 2010; Abranches *et al*., 2004). Then, Pyr can be partitioned depending on oxygen availability, being lactate, formate, acetate and ethanol the main end-products (Brown and Wittenberger, 1972; Takahashi

et al., 1987; 1991; Hillman *et al*., 1994; de Soet *et al*., 2000; Takahashi-Abbe *et al*., 2003). These extracellular compounds being the responsible of acidic activity ascribed to *S. mutans* (Takahashi *et al*., 1991; de Soet *et al*., 2000; Takahashi and Nyvad, 2011).

Using a metabolomic approach, it has been determined that after a Glc rinse there is an increase in levels of Glc-6P, Fru-6P, and Fru-1,6-bisP as well as a decrease in PEP and 3-phosphoglycerate in *S. mutans* (Takahashi and Nyvad, 2011). The activation of key EMP enzymes, such as pyruvate kinase (PK) (Yamada and Carlsson, 1975; Abbe and Yamada, 1982) (which is strictly dependent of Glc-6P activation too) and lactate dehydrogenase (LDH) (Brown and Wittenberger, 1972; Hillman, 1978) would be concomitant to this Fru-1,6-bisP increase. This PK and LDH activation by Fru-1,6-bisP would increase the glycolytic rate, maintaining PEP at very low levels. This metabolic scenario in *S. mutans* (Fig. 9) would allow storage of glycosidic building blocks as IPS. It is well established that ADP-Glc PPases are allosterically regulated by compounds belonging to the principal route for carbon assimilation in the respective organism, being activated by compounds representing high level of energy and inhibited by those that represent low energy levels (Ballicora *et al*., 2003; 2004; Preiss, 2009). Indeed, allosteric compounds reported in this work for *S. mutans* ADP-Glc PPase are in accordance to this statement, since Fru-1,6-bisP (activator) represents sugar availability (as detailed below) and P_i (inhibitor) mirrors low energy. In *Streptococcus*, PEP would be considered as a metabolite representing low energy level which is accumulated in starved cells (Thompson and Thomas, 1977; Takahashi *et al*., 1991; 2010) and is a very strong inhibitor not only of most active conformation of *S. mutans* ADP-Glc PPase, the heterotetrameric GlgC/GlgD, but the Fru-1,6-bisP activated GlgC, as showed in Fig. 9.

In *S. mutans* PEP is used as phosphoryl donor for the sugar PEP-dependent transport system (PTS) during Glc uptake (Thompson and Thomas, 1977; Vadeboncoeur and Pelletier, 1997). In the competitive and constantly changing environment where oral microflora is located PTS is an important tool to economize energy for acquiring and metabolizing sugars. Also, PTS is involved in the control of sugar metabolism in oral streptococci, since it determines the most appropriate energy source and take up the preferred sugar from the external *milieu* at a rate that does not exceed its metabolic capacity (Vadeboncoeur and Pelletier, 1997). The PTS uses PEP in a group translocation process to phosphorylate incoming sugars via a phosphoryl-transfer process (Thompson and Thomas, 1977; Vadeboncoeur and Pelletier, 1997). Then, it can be hypothesized that when sugars are not longer available the PTS will be inactive and the glycolytic flux will become slower, with a concomitant increase in PEP concentration. The whole situation and the PEP increase would inhibit ADP-Glc PPase, thus diminishing the route for IPS synthesis (rather promoting the polysaccharide degradation) in the bacterium (Fig. 9).

Experimental procedures

Chemicals

All protein standards, antibiotics, IPTG, nalidixic acid, oligonucleotides and other chemicals were from the highest quality available obtained from Sigma-Aldrich (St Louis, MO) or similar.

Bacteria and plasmids

Escherichia coli Top 10 F′ cells and pGEM®-T Easy vector were used for cloning purposes. Expression of *glgC* and *glgD* was performed in *E. coli* AC70RI-504, respectively employing the compatible plasmids pMAB6 and pMAB5, which were previously used to characterize ADP-Glc PPase from *Solanum tuberosum* (Iglesias *et al*., 1993). Also, both genes were cloned into pRSFDuet-1 (Novagen) plasmid and expressed using *E. coli* BL21 (DE3). DNA manipulations and *E. coli* cultures as well as transformations were performed according to standard protocols (Sambrook and Russell, 2001).

S. mutans ATCC 25175 planktonic cultures were grown at 37°C in a tryptone-vitamin base medium (3.5% tryptone with 0.04 μg of *p*-aminobenzoic acid ml^{-1}, 0.2 μg of thiamine-HCl ml^{-1}, 1 μg of nicotinamide ml^{-1}, and 0.2 μg of riboflavin ml^{-1}) which was supplemented with 1% (wt/vol) of glucose with 3% CO_2 atmosphere and without stirring. The inoculum consisted of a 12 h culture, adjusted to OD_{600} 0.12. After 16 h growth, cells were harvested by centrifugation, disrupted by sonication and the soluble fraction was used for activity measures and immunodetection experiments.

Amplification of S. mutans *glgC and glgD genes*

The genes coding for both ADP-Glc PPase subunits (GlgC and GlgD) in *S. mutans* were amplified using genomic DNA (from *S. mutans* strain ATCC 25175) as a template and specific primers. The latter were designed using available information in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) for *S. mutans* UA159 (*glgC* GeneID: 1028782; *glgD* GeneID: 102877). Specifically, *SmglgC*fow 5′-AAAGGGCATATGCACATGAAGAATG-3′ (*Nde*I restriction site underlined) and *SmglgC*rev 5′-AAAGGGGAGCTCTC AATCTTCATTTG-3′ (*Sac*I restriction site underlined) were used for *glgC* cloning in pMAB6; whereas *SmglgD*fow: 5′-AAAGGGCATATGATGAAGATTGATAAA-3′ (*Nde*I restriction site underlined) and *SmglgD*rev: 5′-AAAGGGGAGCTC TCATTGAATAATATCCTC-3′ (*Sac*I restriction site underlined) served for *glgD* cloning in pMAB5. In addition, the *glgD* gene was amplified using a second pair of primers in order to later cloning into *Nco*I-*Hind*III restriction sites in pRSFDuet-1 vector: GlgDuetfow: 5′-CCATGGCCATGAAGATTGATAAATA TTC-3′ (*Nco*I restriction site underlined) and GlgDuetrev: 5′-AAGCTTTCATTGAATAATATCCT CAA-3′ (*Hind*III restriction site underlined). PCR reaction mixtures (50 μl) contained

100 ng of genomic DNA, 2 pg of each primer; 0.2 mM of each dNTP; 1.5 mM Mg^{2+} and 1U *Taq* DNA polymerase (Fermentas). For both genes amplification 30 cycles of PCR were performed under conditions: denaturation at 94°C for 1 min; annealing at 50°C for 1 min and extension at 72°C for 1.5 min with a final extension at 72°C for 10 min. PCR reaction mixtures were electrophoretically defined in an 1% (wt/vol) agarose gel and purified with Wizard SV gel & PCR Clean Up system (Promega) according to the manufacturer's instructions.

Homology modelling

Modelling of the GlgC and GlgD subunits from the *S. mutans* ADP-Glc PPase enzyme was performed using the program Modeller 9v1 (Sali and Blundell, 1993). For that purpose, the structure was modelled using the structure of the ADP-Glc PPase from *A. tumefaciens* (PDB code 3BRK), and the structure of potato tuber ADP-Glc PPase in complex with sulphates in the putative regulatory site. The *A. tumefaciens* structure is particularly good for this, since it is from a bacterial source like *S. mutans* and the potato tuber structure provides information about where the sulphate ligands would go. Then, the co-ordinates of these ADP-Glc PPases provided the structural information to build the N-terminal domains of *S. mutans* GlgC and GlgD, where is the pocket for substrate binding. The templates were manually structurally aligned to each other before the sequence alignment was performed with *S. mutans* GlgC or GlgD targets. Reliability of the models was evaluated using the program Verify3D (Luthy *et al.*, 1992). This validation was already good and further iterations of the alignment were minimal.

Cloning of glgC and glgD genes

Amplified genes were cloned into the T-tailed plasmid pGEM-TEasy and used to transform *E. coli* Top 10 F' cells. Gene identities were confirmed by DNA sequencing. Genes *glgC* and *glgD* were then respectively subcloned into the pMAB6 and pMAB5 compatible expression vectors (Yep *et al.*, 2004), using *Nde*I and *Sac*I restriction sites to obtain [pMAB6/*glgC*] and [pMAB5/*glgD*] constructs. The vector pMAB5 is a derivative of the pMON17335 and pACYC177 with a *tac* promoter and a phage T7 gene10 leader translation enhancer. Also, pMAB6 is a derivative of pMON17336 and pBR327 with a PrecA and a gene10 leader translation enhancer expression cassette (Iglesias *et al.*, 1993). As well, pRSFDuet-1 vector, which is designed for the coexpression of two target proteins, was employed to overexpress *glgC* and *glgD* genes in *E. coli* BL21 (DE3). It has two multi cloning sites (MCS) each of which is preceded by a T7 promoter. Thus, *glgC* from *S. mutans* was cloned in the *Nde*I and *Sac*I sites from MCS2 and *glgD* was inserted between *Nco*I and *Hind*III sites in the MCS1 of pRSFDuet-1, to obtain expression vectors [pDUET/*glgC*], [pDUET/*glgD*] and [pDUET/*glgCD*].

Enzyme expression and purification

Escherichia coli AC7OR1-504 cells lacking endogenous ADP-Glc PPase activity were transformed with either [pMAB6/*glgC*] or [pMAB5/*glgD*] to respectively express GlgC or GlgD proteins. Also, for expression of GlgC/GlgD cells were co-transformed with [pMAB6/*glgC*] and [pMAB5/*glgD*] plasmids (Iglesias *et al.*, 1993). Transformed cells were grown in 1 l of LB (Lisogeny broth) medium at 37°C, 200 r.p.m., until the OD_{600} of 1.1–1.3. Recombinant protein expression was induced with 0.4 mM IPTG for GlgD or 0.5 μg ml^{-1} nalidixic acid for GlgC (or both inducers together for GlgC/GlgD). Alternatively, GlgC, GlgD or GlgC/GlgD proteins were obtained in *E. coli* BL21 (DE3) transformed with [pDUET/*glgC*], [pDUET/*glgD*] or [pDUET/*glgCD*] vectors respectively. Cells were grown in LB medium and cultures were induced with 0.5 mM IPTG. In all cases, proteins were expressed for 16 h at 20°C and harvested by centrifugation at 5000 r.p.m. for 10 min at 4°C. Then, cells were resuspended in 5 ml of *buffer A* [50 mM MOPS, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, and 5% (wt/vol) sucrose] per gram of cells and disrupted by sonication on ice (8 pulses for 30 s with 60 s intervals). Crude extracts were the supernatants obtained after centrifugation at 16000 r.p.m. for 20 min at 4°C.

GlgC and GlgC/GlgD proteins overexpressed with pRSFDuet-1 vector were purified to near homogeneity using the following method. Crude extracts were loaded onto a 10 ml DEAE-Sepharose Fast Flow weak anion exchange column (GE Healthcare) equilibrated with *buffer A* and eluted with a 0–0.5 M linear gradient of NaCl (20 column volumes, 1 ml min^{-1} flow rate). Active fractions were pooled and precipitated with ammonium sulphate at 70% saturation. Proteins were resuspended in *buffer C* (*buffer A* plus 1 M ammonium sulphate) and loaded onto 2 ml Phenyl-Sepharose hydrophobic resin in a C10/10 column (GE Healthcare) equilibrated with *buffer C*. Proteins were eluted with a linear gradient of ammonium sulphate (20 column volumes, 1 to 0 M, 0.5 ml min^{-1} flow rate). Fractions containing the highest activity were pooled, desalted, and concentrated. An additional step of purification was conducted using an 1 ml Resource Q column (GE Healthcare) equilibrated with *buffer A* and eluted with a 0–0.5 M linear gradient of NaCl (20 column volumes; 2 ml min^{-1} flow rate). Highly active fractions were pooled, desalted and concentrated. Samples were supplemented with 20% (wt/vol) glycerol and stored at –80°C, conditions under which GlgC and GlgC/GlgD remained fully active during for at least 3 months.

Iodine staining

A modified procedure for iodine staining of cells growing in liquid media was used, according to (Ballicora *et al.*, 2007; Kuhn *et al.*, 2010). Briefly, transformed *E. coli* AC7OR1-504 cells harbouring *S. mutans glgC*, *glgD* or both genes were inoculated onto 3 ml of LB medium and grown at 37°C until they reached an OD_{600} ~ 0.8. Protein expression was induced for 3 h as described *ut supra*. Afterwards, glucose was added to a final concentration of 0.2% (wt/vol) and extended the incubation for 1 h. An aliquot of 0.1 ml was withdrawn and centrifuged in a 1.5 ml microcentrifuge tube at 14 000 r.p.m. for 5 min. Supernatant was carefully aspirated to remove all liquid, leaving a compact pellet at the bottom of the tube. The microcentrifuge tube was turned upside down, and an iodine crystal was positioned in the cap of the tube (base), and the tube was closed. In 5 min, iodine vapour stained the cell pellet (top).

Protein measurement

Protein concentration was determined by the modified Bradford assay (Bradford, 1976) using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis and Western blotting

Recombinant proteins and purification fractions were defined by SDS-PAGE according to Laemmli (1970). Gels were loaded with 5 to 50 μg of protein per well and stained with Coomassie brilliant blue. Western blotting was performed after standard techniques (Sambrook and Russell, 2001). Proteins in the gel were blotted onto PVDF membranes using a Mini-PROTEAN II (Bio-Rad) apparatus. The membrane was blocked 2 h at 4°C and subsequently incubated overnight with primary antibody at room temperature. Then, membranes were incubated with Alexa Fluor 647 goat anti-rabbit (H+L) (Invitrogen) during 1 h. Detection was carried out by scanning membranes at 650 nm with the Typhoon 9400 equipment.

Antibodies raised against GlgC and GlgD from *G. stearothermophilus* were produced in our lab according to established methods (Vaitukaitis, 1981) and used as primary antibodies. They were purified from rabbit sera by consecutive precipitation steps with ammonium sulphate 50% and 33% (twice) saturation solutions. After that, antibodies were resuspended in TBS buffer (Tris-HCl pH 8.0, NaCl 150 mM) and desalted using a ultrafiltration device with a 30 kDa cut-off (Amicom).

Molecular mass determination

The molecular mass of the GlgC and GlgC/GlgD proteins were determined by gel filtration on Superdex 200 using a Tricorn 5/200 column (GE Healthcare). It was used a Gel Filtration Calibration Kit–High Molecular Weight (GE Healthcare) with protein standards including thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (44 kDa). The column void volume was determined using dextran blue loading solution (Promega).

Enzyme assays

Synthesis direction, radiometric. The method of Yep (Yep *et al.*, 2004) was used to measure synthesis of ADP-[^{14}C]Glc from [^{14}C]Glc-1P and ATP. Unless otherwise specified, the standard reaction mixture contained 100 mM MOPS buffer (pH 8.0), 10 mM $MgCl_2$, 1 mM [^{14}C]Glc-1P (100–1000 cpm nmol^{-1}), 2 mM ATP, 0.5 mU μl^{-1} inorganic pyrophosphatase, and 0.2 mg ml^{-1} bovine serum albumin plus enzyme in a total volume of 0.2 ml. Reaction mixtures were incubated for 10 min at 37°C and terminated by 1 min heating in a boiling-water bath. ADP-[^{14}C]Glc formed during the reaction was measured as previously described (Yep *et al.*, 2004).

Synthesis direction, colorimetric. Synthesis of ADP-Glc was assayed by following formation of P_i (after hydrolysis of PP_i by inorganic pyrophosphatase) by the highly sensitive colorimetric method previously described (Fusari *et al.*, 2006). The reaction mixture contained 100 mM MOPS (pH 8.0), 10 mM $MgCl_2$, 1.5 mM ATP, 0.2 mg ml^{-1} bovine serum albumin, 0.5 Mu μl^{-1} yeast inorganic pyrophosphatase and a proper enzyme dilution. Assays were initiated by addition of 0.5 mM Glc-1P for GlgC/GlgD or 2 mM Glc-1P for GlgC, in a total volume of 50 μl. Reaction mixtures were incubated for 10 min at 37°C and terminated by the addition of the Malachite Green reactive (Fusari *et al.*, 2006). The complex formed with the released P_i was measured at 630 nm with an ELISA EMax detector (Molecular Devices). $NaPP_i$ was used as standard for the whole procedure.

One unit (U) of enzyme activity is equal to 1 μmol of product formed per minute under the respective assay conditions above specified.

Determination of optimal pH for activity

Bis-Tris-propane [2,2′-(Propane-1,3-diyldiimino)bis[2-(hydroxymethyl)propane-1,3-diol] (Sigma), which has a wide buffering range (from pH 6.0 to pH 10.0), was used to calculate the optimal pH for *S. mutans* ADP-Glc PPase activity. Assays were conducted in the ADP-Glc synthesis direction using the colorimetric method.

Calculation of kinetic constants

Saturation curves were performed by assaying enzyme activity at different concentrations of the variable substrate or effector and saturating levels of the other effectors. The experimental data were plotted as enzyme activity (U mg^{-1}) versus substrate (or effector) concentration (mM), and kinetic constants were determined by fitting the data to the Hill equation as described elsewhere (Ballicora *et al.*, 2007). Fitting was performed with the Levenberg–Marquardt non-linear least-squares algorithm provided by the computer program Origin™. Hill plots were used to calculate the Hill coefficient (n_H), the maximal velocity (V_{max}), and the kinetic constants that correspond to the activator, substrate or inhibitor concentrations giving 50% of the maximal activation ($A_{0.5}$), velocity ($S_{0.5}$) or inhibition ($I_{0.5}$). All kinetic constants are the mean of at least three independent sets of data, which were reproducible within ± 10%. Similar results were obtained with four different preparations of purified GlgC and GlgC/GlgD enzymes.

Phylogenetic analysis

Amino acidic sequences of different GlgC and GlgD polypeptides belonging to ADP-Glc PPases from Firmicutes were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/). They were filtered to remove duplicates and near duplicates (i.e. mutants and strains from same species). After a preliminary alignment, constructed using the ClustalW multiple sequence alignment server (http://www.genome.jp/tools/clustalw/) (Jeanmougin *et al.*, 1998), sequences with a wrong annotation or truncated were also eliminated manually. Sequences having lower than 30% identity to any other and did not have all the characterized critical motifs and catalytic residues were discarded. After this, sequences were chosen to represent most of the taxonomic groups from Firmicutes. Sequences were classified into different groups using taxo-

nomic data provided by the NCBI as depicted in Table S1. Once we settle on the sequences to use, they were aligned using the program T-Coffee (Notredame *et al*., 2000). A manual inspection was performed to guarantee that all known conserved regions (i.e. catalytic residues) were properly aligned. Trees were constructed by maximum likelihood with the program PhyML (Gouy *et al*., 2010) incorporated into Seaview. Confidence coefficients for the tree branches were obtained and plotted. Finally, the tree was prepared with the FigTree 1.3 program (http://tree.bio.ed.ac.uk/) (Guindon and Gascuel, 2003).

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Conflict of interest

The authors declare no conflict of interest.

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