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GlgS, described previously as a glycogen synthesis control protein, negatively regulates motility and biofilm formation in *Escherichia coli*

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Escherichia coli glycogen metabolism involves the regulation of *glgBXCAP* operon expression and allosteric control of the GlgC [ADPG (ADP-glucose) pyrophosphorylase]-mediated catalysis of ATP and G1P (glucose-1-phosphate) to ADPG linked to glycogen biosynthesis. *E. coli* glycogen metabolism is also affected by *glgS*. Though the precise function of the protein it encodes is unknown, its deficiency causes both reduced glycogen content and enhanced levels of the GlgC-negative allosteric regulator AMP. The transcriptomic analyses carried out in the present study revealed that, compared with their isogenic BW25113 wild-type strain, *glgS*-null (Δ*glgS*) mutants have increased expression of the operons involved in the synthesis of type 1 fimbriae adhesins, flagella and nucleotides. In agreement, Δ*glgS* cells were hyperflagellated and hyperfimbriated, and displayed elevated swarming motility; these phenotypes all reverted to the wild-type by ectopic *glgS* expression. Also, Δ*glgS* cells accumulated high colanic acid content and displayed increased ability to form biofilms on polystyrene surfaces. F-driven conjugation based on large-scale interaction studies of *glgS* with all the non-essential genes of *E. coli* showed that deletion of purine biosynthesis genes complement the glycogen-deficient, high motility and high biofilm content phenotypes of Δ*glgS* cells. Overall the results of the present study indicate that glycogen deficiency in Δ*glgS* cells can be ascribed to high flagellar propulsion and high exopolysaccharide and purine nucleotides biosynthetic activities competing with GlgC for the same ATP and G1P pools. Supporting this proposal, glycogen-less Δ*glgC* cells displayed an elevated swarming motility, and accumulated high levels of colanic acid and biofilm. Furthermore, *glgC* overexpression reverted the glycogen-deficient, high swarming motility, high colanic acid and high biofilm content phenotypes of Δ*glgS* cells to the wild-type. As on the basis of the present study GlgS has emerged as a major determinant of *E. coli* surface composition and because its effect on glycogen metabolism appears to be only indirect, we propose to rename it as ScoR (surface composition regulator).

Key words: biofilm, exopolysaccharide, flagellar motility, GlgS, glycogen, growth regulation, large-scale genetic interaction.

INTRODUCTION

Glycogen is a branched homopolysaccharide of α-1,4-linked glucose subunits with α-1,6-linkages at the branching points that is synthesized by GlgA (glycogen synthase) using ADPG (ADP-glucose) as the glucosyl moiety donor. Glycogen accumulation in *Escherichia coli* is an energy (ATP)-consuming process that occurs when cellular carbon sources are in excess, but there is a deficiency of other nutrients. The exact role of this reserve polysaccharide in bacteria is still not well defined, but several works have linked glycogen metabolism to environmental survival, intestine colonization and virulence [1–5]. In this context, a recent study has shown that *E. coli* internal glycogen, rather than external glucose, may provide for the primary energy source during bacterial adaptation to fresh conditions before initiating active proliferation [6].

Regulation of bacterial glycogen biosynthesis involves a complex assemblage of factors that are adjusted to the nutritional status of the cell [7,8]. At the level of enzyme activity for instance, glycogen biosynthesis is subjected to the allosteric regulation of GlgC (ADPG pyrophosphorylase), which produces ADPG from ATP and G1P (glucose-1-phosphate) [9]. In general, the activators of GlgC in heterotrophic bacteria are key metabolites whose presence indicates high levels of carbon and energy within the cell, whereas inhibitors of this enzyme are indicators of low metabolic energy levels. In the case *E. coli*, fructose 1,6-bisphosphate activates GlgC, whereas AMP acts as an important inhibitor [9]. At the transcriptional level a recent study has shown that all *E. coli* glycogen synthetic and breakdown genes are organized in a single *glgBXCAP* transcriptional unit forming part of both the RelA and PhoP–PhoQ regulons [10]. In *E. coli*, glycogen accumulation is positively affected by the product of the *glgS*

Abbreviations used: ADPG; ADP-glucose; Ag43; antigen 43; CsrA; carbon storage regulator; EPS; exopolysaccharide; GalU; UTP–glucose-1-phosphate uridylyltransferase; GlgA; glycogen synthase; GlgC; ADPG pyrophosphorylase; G1P; glucose-1-phosphate; (p)ppGpp; guanosine tetra(penta) phosphate; LB; Luria–Bertani; RpoS; RNA polymerase sigma factor; ScoR; surface composition regulator; trp; tryptophan synthase; WT; wild-type.

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gene [7,11–13], a hydrophilic and highly charged 7.9 kDa protein with no significant homology outside the Enterobacteriaceae family [14,15]. *E. coli glgS* expression is negatively regulated by the global post-transcriptional regulator CsrA (carbon storage regulator) [16]. Moreover, it exhibits strong stationary-phase induction [11,17], being positively regulated by the general stress regulator RpoS (RNA polymerase sigma factor) [11], the stringent response regulator (p)ppGpp [guanosine tetra(penta) phosphate] [18,19] and the RNA chaperone Hfq, whose translation is in turn inhibited by CsrA [20,21].

Despite characterization of several of the enzymes involved in glycogen synthesis, the precise function of GlgS is still poorly resolved. A previous study suggested that GlgS might be a site for primary sugar attachment during the glycogen initiation process [14]. However, this hypothesis was weakened by the observation that *Agrobacterium tumefaciens* GlgA does not require additional proteins for glycogen priming [22]. More recently, we found that *E. coli glgS* deletion (Δ*glgS*) mutants accumulate high levels of AMP, the negative allosteric regulator of GlgC [7].

To gain insight into the cellular function(s) of GlgS, we conducted a transcriptomic analysis of *E. coli* BW25113 Δ*glgS* cells. Comparison with the isogenic BW25113 WT (wild-type) strain revealed that *glgS* expression negatively affects the expression of the genes involved in the formation of cell surface organelles, including type 1 fimbriae and flagella, and in the synthesis of purines and pyrimidines. Consequently, Δ*glgS* mutants showed increased flagella and fimbriae production, were hypermotile, and produced more biofilm than the WT cells. These results, and those obtained from F-driven conjugation on the basis of large-scale predictions of genomic interactions between *glgS* and the 3984 non-essential genes of *E. coli*, indicated that GlgS exerts a negative effect on flagellar propulsion and biofilm polysaccharide production. Both of these processes compete with GlgC-controlled glycogen biosynthesis for the same ATP and G1P pools. On the basis of the observations reported in the present study, we propose that GlgS acts as a major negative regulator of processes involved in *E. coli* propulsion, adhesion and synthesis of biofilm EPSs (exopolysaccharides), and that net glycogen accumulation represents the major use for the surplus ATP and G1P of the above processes under conditions of carbon excess. Because GlgS emerges now as a major determinant of *E. coli* surface composition, and because its effect on glycogen metabolism appears to be only indirect, we propose to rename it as ScoR (surface composition regulator).

EXPERIMENTAL

Bacterial strains, plasmids and culture conditions

The strains, mutants and plasmids used in the present study are shown in Table 1. *E. coli* K-12 derivative BW25113 single-gene knockout mutants were obtained from the Keio collection [23]. *LacZY* transcriptional fusions were constructed and verified as reported in Montero et al. [10]. Double knockout mutants were constructed using single knockout mutants from the Keio collection. The kanamycin resistance cassette from the recipient strain was removed by using the temperature-sensitive plasmid pCP20 that carries the FLP recombinase [24]. The deletion from the donor strain was then P1-transduced [25] into the recipient strain. Kanamycin-containing LB (Luria–Bertani) plates were used to select the double mutants, whose deletions were verified by PCR. Cells expressing *glgC* and *glgS in trans* were obtained by incorporation of *glgC*- and *glgS*-expression vectors from the ASKA library [26]. Unless otherwise indicated, cells were grown at 37 °C with rapid gyratory shaking in liquid Kornberg medium (1.1 % K_2HPO_4, 0.85 % KH_2PO_4 and 0.6 % yeast extract; Difco) supplemented with 50 mM glucose and the appropriate selection antibiotic, after inoculation with 1 volume of an overnight culture for 100 volumes of fresh medium.

Analytical procedures

Bacterial growth was followed spectrophotometrically by measuring the absorbance at 600 nm. Cells from cultures entering the stationary phase were centrifuged at 4400 ***g*** for 15 min at 4 °C, rinsed with fresh Kornberg medium, resuspended in 40 mM Tris/HCl (pH 7.5) and disrupted by sonication prior to quantitative measurement analyses of protein and glycogen contents. β-Galactosidase activity was measured and reported graphically as described by Miller [27]. Protein content was measured by the Bradford method using a Bio-Rad Laboratories prepared reagent. Qualitative analysis of the glycogen content of cells cultured on solid glucose Kornberg medium was carried out using the iodine-staining technique [28]. Quantitative glycogen measurement analyses were carried out using an amyloglucosidase-based test kit (Boheringer Manheim). Extraction and measurement of colanic acid content was carried out as described by Obadia et al. [29].

Electron microscopy examination of type 1 fimbriae and flagella

Cells entering the stationary phase were centrifuged at 4000 ***g*** for 15 min at 4 °C. The collected cells were rinsed twice with liquid Kornberg/glucose medium, resuspended using a PBS solution and fixed in 1 % of osmium tetroxide for 15 min before being applied to 200-mesh Formvar-coated copper specimen grids. These preparations were negatively stained with 2 % (w/v) phosphotungstic acid before examination in an EFTEM Zeiss Libra 120 transmission electron microscope.

Microarrays

Δ*glgS* and WT cells were grown in 20 ml of liquid Kornberg/glucose medium at 37 °C in aerobic conditions under shaking and harvested at the onset of the stationary phase. The cultures were then centrifuged (4400 ***g*** for 5 min at 4 °C), and the obtained pellets were frozen in liquid nitrogen and stored at − 80 °C until needed. Total RNA was extracted using the TRIzol reagent method as described by Toledo-Arana et al. [30]. Fluorescently labelled cDNA for microarray hybridizations were obtained by using the SuperScript Indirect cDNA Labelling System (Invitrogen) following the manufacturer's instructions. The hybridization experiment was performed on the Agilent *E. coli* microarray 8×15K (G4813A-020097, Agilent). Three independent biological replicates were hybridized for Δ*glgS* and WT cells. The expression data were statistically analysed using the LIMMA Package [31]. Statistically differentially expressed genes were selected on the basis of their *P* values ($P < 0.05$ determined by Student's *t* test) and the fold changes in Δ*glgS* cells compared with the WT. Functional characterization of the differentially expressed genes was done using the KEGG (http://www.genome.jp/kegg) and RegulonDB (http://regulondb.ccg.unam.mx) databases.

Motility tests

Swarm motility plates were prepared as reported by Niu et al. [32] on LB plates supplemented with 0.6 % Bacto agar and 0.01 %

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Table 1 The bacterial strains and plasmids used in the present study

Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance.

	Description	Source
Bacteria		
W	A.T.C.C. 9637	[78]
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Keio collection [23]
Δ*wzc*	BW25113 Complete *wzc* replaced by a Km^R cassette	Keio collection [23]
Δ*fimA*	BW25113 Complete *fimA* replaced by a Km^R cassette	Keio collection [23]
Δ*flhC*	BW25113 Complete *flhC* replaced by a Km^R cassette	Keio collection [23]
Δ*fliA*	BW25113 Complete *fliA* replaced by a Km^R cassette	Keio collection [23]
Δ*glgS*	BW25113 Complete *glgS* replaced by a Km^R cassette	Keio collection [23]
Δ*glgS**	BW25113 Δ*glgS* where the Km^R was removed using FRT sites	The present study
Δ*glgC*	BW25113 Complete *glgC* replaced by a Km^R cassette	Keio collection [23]
Δ*glgC**	BW25113 Δ*glgC* where the Km^R was removed using FRT sites	The present study
Δ*glgA*	BW25113 Complete *glgA* replaced by a Km^R cassette	Keio collection [23]
Δ*purM*	BW25113 Complete *purM* replaced by a Km^R cassette	Keio collection [23]
Δ*purL*	BW25113 Complete *purL* replaced by a Km^R cassette	Keio collection [23]
Δ*galU*	BW25113 Complete *galU* replaced by a Km^R cassette	Keio collection [23]
glgB::lacZY	BW25113 *glgB::lacZY* transcriptional fusion	[10]
glgX::lacZY	BW25113 *glgX::lacZY* transcriptional fusion	[10]
glgC::lacZY	BW25113 *glgC::lacZY* transcriptional fusion	[10]
glgA::lacZY	BW25113 *glgA::lacZY* transcriptional fusion	[10]
glgP::lacZY	BW25113 *glgP::lacZY* transcriptional fusion	[10]
fliA::lacZY	BW25113 *fliA::lacZY* transcriptional fusion	The present study
flhC::lacZY	BW25113 *flhC::lacZY* transcriptional fusion	The present study
fimA::lacZY	BW25113 *fimA::lacZY* transcriptional fusion	The present study
ycgR::lacZY	BW25113 *ycgR::lacZY* transcriptional fusion	The present study
trpE::lacZY	BW25113 *trpE::lacZY* transcriptional fusion	The present study
flgB::lacZY	BW25113 *flgB::lacZY* transcriptional fusion	The present study
motA::lacZY	BW25113 *motA::lacZY* transcriptional fusion	The present study
lrhA::lacZY	BW25113 *lrhA::lacZY* transcriptional fusion	The present study
Δ*glgS glgB::lacZY*	BW25113 *glgB::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS glgX::lacZY*	BW25113 *glgX::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS glgC::lacZY*	BW25113 *glgC::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS glgA::lacZY*	BW25113 *glgA::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS glgP::lacZY*	BW25113 *glgP::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS fliA::lacZY*	BW25113 *fliA::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS flhC::lacZY*	BW25113 *flhC::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS fimA::lacZY*	BW25113 *fimA::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS ycgR::lacZY*	BW25113 *ycgR::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS trpE::lacZY*	BW25113 *trpE::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS flgB::lacZY*	BW25113 *flgB::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS motA::lacZY*	BW25113 *motA::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS lrhA::lacZY*	BW25113 *lrhA::lacZY* transcriptional fusion in Δ*glgS**	The present study
Δ*glgS*Δ*purM*	BW25113 Δ*purM* P1 phage transduced in Δ*glgS**	The present study
Δ*glgS*Δ*purL*	BW25113 Δ*purL* P1 phage transduced in Δ*glgS**	The present study
Δ*glgS*Δ*fimA*	BW25113 Δ*fimA* P1 phage transduced in Δ*glgS**	The present study
Δ*glgS*Δ*flhC*	BW25113 Δ*flhC* P1 phage transduced in Δ*glgS**	The present study
Δ*glgS*Δ*wzc*	BW25113 Δ*wzc* P1 phage transduced in Δ*glgS**	The present study
Δ*glgS*Δ*galU*	BW25113 Δ*galU* P1 phage transduced in Δ*glgS**	The present study
Δ*glgC*Δ*galU*	BW25113 Δ*galU* P1 phage transduced in Δ*glgC**	The present study
Plasmid	Description	Source
pCP20	Plasmid expressing FLP recombinase, Amp^R, used for removal of Km^R cassettes	[79]
pKG137	Plasmid including *lacZY* and Km^R cassette, used for construction of transcriptional fusions	[10]
pCA24N*glgC*	Plasmid used for overexpression of *glgC*, Cm^R	ASKA collection [26]
pCA24N*glgS*	Plasmid used for overexpression of *glgS*, Cm^R	ASKA collection [26]

Tween 80. After an overnight incubation at 28 °C, the plates were inspected for bacterial growth and motility.

Crystal Violet biofilm assay

This assay was adapted from that described by Pratt and Kolter [33]. Cells were grown in polystyrene 96-well microtiter plates (catalogue number 82.1581.001, Sarstedt) at 28 °C for 48 h without shaking in Kornberg/glucose liquid medium. Microtiter plates were rinsed thoroughly with water, and the cells were stained with 1 % Crystal Violet for 20 min, rinsed again with water and dried. The retained Crystal Violet was then solubilized by the addition of 100 μl of ethanol/acetone (70:30) (for further details, see Lehnen et al. [34]) and quantified by spectrometry at 595 nm [35]. The biofilm content was normalized by cell growth (turbidity at 620 nm) as described by Zhang et al. [36].

High-throughput generation of a double-mutant library for the identification of genes whose deletions affect glycogen accumulation in Δ*glgS* cells

High-throughput generation of a library of double mutant Δ*glgS* cells crossed with the 3985 single-gene knockout mutants of

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non-essential genes of the Keio collection was carried out essentially as described by Typas et al. [37] except that the pseudo-Hfr *glgS* mutant belonging to the ASKA single-gene deletion library marked with the *cat* (chloramphenicol acetyltransferase) chloramphenicol-resistance gene was mated on agar plates to the Keio recipient strains. This method permits the systematic generation and array of double mutants on a solid medium in high-density arrays. The library thus obtained was screened in solid Kornberg medium supplemented with 50 mM glucose for altered glycogen content using the iodine-staining method [28]. In the presence of iodine vapour, 'glycogen-excess' mutants stain darker than their brownish parental cells, whereas 'glycogen-deficient' mutants stain yellow.

RESULTS AND DISCUSSION

Transcriptome profile of BW25113 Δ*glgS* cells

To investigate the cellular mechanisms associated with a reduction of glycogen content in *E. coli* Δ*glgS* cells [7,11,12] we compared the genome-wide expression profiles of the *E. coli* K-12 strain derivative BW25113 (WT) and its isogenic Δ*glgS* mutant in cells entering the stationary phase using whole-genome microarrays as described in the Experimental section. Genes with differential expression were classified according to the KEGG and RegulonDB databases. Briefly, our transcriptome profiling analysis revealed that 129 genes showed statistically significant changes in transcript level in Δ*glgS* cells when compared with the WT cells. Among this population 94 genes were up-regulated (Table 2) and 35 genes were down-regulated (Table 3) in Δ*glgS* cells. To further examine the distribution of the genes with regard to their functions, the genes with transcript level changes were classified into COGs (clusters of orthologous groups) [38] (Figure 1). As shown in Table 2, the type 1 fimbriae operon, the *flhDC* master operon encoding the master transcriptional regulator of the flagellar regulon, the FlhDC-controlled Class II operons required for the structural assembly of the hook and the basal body of the flagellum, the FliA (σ^{28})-controlled Class III operons required for flagellar motility and chemotaxis, as well as the FlhDC-controlled genes *yhjH* and *ycgR* (the latter encoding a PilZ-domain protein that interacts with the flagellar motor to promote motile-to-sessile transitions in response to increased c-di-GMP concentrations [39]) were all up-regulated in Δ*glgS* cells. Together these genes accounted for nearly 60% of the genes whose expression is up-regulated in Δ*glgS* cells. Furthermore, five out of the six operons involved in the *de novo* synthesis of pyrimidines [*carAB* (carbamoyl-phosphate synthase), *pyrLBI*, *pyrC* (dihydroorotase), *pyrD* (dihydroorotase dehydrogenase) and *pyrF* (orotidine 5'-phosphate decarboxylase)], two operons involved in the salvage pathway of UMP synthesis from pyrimidine bases and nucleosides [*codBA* (cytosine deaminase/permease) and *upp* (uracil phosphoribosyltransferase)] and three operons involved in *de novo* synthesis of purines [*purHD*, *purMN* and *guaBA* (GMP synthase)] were all up-regulated in Δ*glgS* cells when compared with the WT cells (Table 2). Analyses of the expression of chromosomal *lacZY* transcriptional fusions of some of the identified genes on both WT and Δ*glgS* cells (Supplementary Figure S1 at http://www.biochemj.org/bj/452/bj4520559add.htm) validated the results of our array analyses, the results of which are shown in Tables 2 and 3.

It is noteworthy that our array analyses did not reveal significant differences in *glgBXCAP* transcript levels between the Δ*glgS* and WT cells, which was further confirmed by the use of *glgB::lacZY* transcriptional fusions (Supplementary Figure S1) and Western blot analyses of GlgC (results not shown) on WT and Δ*glgS* cells. This indicates that the reported positive effect of GlgS on *E. coli* glycogen accumulation [7,11–13] is not ascribed to changes in the *glgBXCAP* expression levels in *E. coli*.

GlgS negatively affects type 1 fimbriation and flagella production in *E. coli*

The transcript profile analyses shown in Tables 2 and 3 suggest that GlgS negatively regulates *E. coli* type 1 fimbriation and the synthesis of flagella. To evaluate this possibility, we carried out electron microscopy analyses of BW25113 WT and Δ*glgS* cells entering the stationary phase. As shown in Figure 2, these analyses revealed that Δ*glgS* cells were hyperflagellated and hyperfimbriated when compared with the WT cells, the overall data thus confirming the idea that GlgS exerts a negative effect on the synthesis of type 1 fimbriae and flagella in *E. coli*.

GlgS exerts a negative effect on *E. coli* swarming motility

Swarming is a flagellum-dependent form of bacterial motility that facilitates the migration of bacteria on viscous substrates, such as semisolid agar surfaces. To swarm, cells first differentiate into a specialized state (swarmer cells) characterized by an increase in flagellum number and the elongation of cells [40,41]. Synthesis of the flagellum and its related components in *E. coli* involves 14 operons and over 50 genes (most of them included in Table 2) whose expression is under a hierarchical control system wherein *flhDC* acts as the master regulatory operon [42]. Inoue et al. [43] have provided evidence that expression of type 1 fimbriae genes is also required for swarming motility in *E. coli*.

Our transcriptome and electron microscopy analyses showing that production of both flagella and type 1 fimbriae are enhanced in Δ*glgS* cells (Figure 2 and Table 2) predicting that GlgS exerts a negative effect on swarming motility. We thus compared the swarming motility between BW25113 WT and Δ*glgS* cells in soft Tween swarm agar plates (see the Experimental section). We also included as control Δ*glgS*Δ*flhC* and Δ*glgS*Δ*fliA* cells impaired in the formation of flagella. As shown in Figure 3(A), BW25113 WT cells exhibited very low swarming, in agreement with previous observations reported by other authors [44]. In sharp contrast, their isogenic Δ*glgS* cells displayed a 'high-swarming motility' (hypermotile) phenotype (Figure 3A). Moreover, as seen in the same Figure, this hypermotile phenotype could be reverted to the WT by ectopic expression of *glgS*, therefore ruling out pleiotropic effects owing to the Δ*glgS* mutation. Furthermore, the introduction of Δ*flhC*- or Δ*fliA*-null alleles into the hypermotile Δ*glgS* cells resulted in cells that could no longer swarm, the overall data thus supporting the idea that GlgS exerts a negative effect on swarming motility. The proposed negative effect of GlgS on *E. coli* swarming motility was further supported by analysing the effects of *glgS* overexpression in the 'high-swarming motility' *E. coli* W strain [45]. As shown in Figure 3(B), the ectopic expression of *glgS* in W cells drastically reduced their intrinsic hypermotility in swarming plates.

GlgS negatively affects biofilm formation in *E. coli*

Gram-negative bacteria such as *E. coli* are capable of undergoing a shift from free-living (planktonic) to a sessile growth form known as a biofilm [46]. Biofilms are surface-attached microbial communities included in a self-produced EPS matrix that possess phenotypic and biochemical properties distinct from free-living planktonic cells. Developmental steps in biofilm formation

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Table 2 Genes showing significantly enhanced transcript levels in *E. coli* BW25113 Δ*glgS* mutants

OMP, orotidine 5′-phosphate.

(a) Polycistronic operons

Gene	Fold change*	Function (KEGG entry)†
carAB		
carA	1.63	Carbamoyl-phosphate synthetase, glutamine (b0032)
carB	1.77	Carbamoyl-phosphate synthase large subunit (b0033)
codBA		
codB	1.54	Cytosine permease/transport (b0336)
codA	2.14	Cytosine deaminase (b0337)
cusCFBA		
cusC	33.35	Silver and copper efflux, outer membrane lipoprotein component (c_0658)
cusF	22.12	Silver- and cuprous copper-binding protein, periplasmic; efflux metallochaperone (b0573)
cusB	42.06	Silver and copper efflux, membrane fusion protein; confers copper and silver resistance (b0574)
cusA	10.46	Silver and copper efflux, membrane transporter (c_0661)
cusRS		
cusR	6.64	Two-component system response regulator of the *cusCFBA* operon (b0571)
cusS	5.18	Two-component system regulator of the *cusCFBA* operon, copper ion sensor (b0570)
cvpA-purF		
cvpA	1.59	Membrane protein required for colicin V production (b2313)
purF	1.55	Amidophosphoribosyltransferase, purine synthesis (b2312)
dcuB-fumB		
dcuB	1.53	Anaerobic dicarboxylate transport (b4123)
fimAICDFGH		
fimA	2.83	Fimbrin type 1, major structural subunit; phase variation (b4314)
fimI	1.96	Required for fimbriae biosynthesis, FimA homologue (b4315)
fimC	3.13	Periplasmic chaperone for type 1 fimbriae; FimCD chaperone-usher transport (b4316)
fimD	2.05	Outer membrane protein involved in export and assembly of type 1 fimbrial subunits; FimCD chaperone-usher transport (b4317)
fimF	2.05	Fimbrin type 1 minor component; fimbrial morphology and assembly (b4318)
fimG	2.60	Fimbrin type 1 minor component; fimbriae length (b4319)
fimH	1.61	Minor type 1 fimbrial subunit; membrane-specific adhesin; mediates mannose-binding to host surfaces (b4320)
flgAMN		
flgA	3.76	Flagellar synthesis; assembly of basal-body periplasmic P ring (b1072)
flgM	2.62	Anti-σ^{28} (FliA) factor; regulator of FlhD (b1071)
flgN	2.03	Initiation of flagellar filament assembly (b1070)
flgBCDEFGHIJ		
flgB	10.83	Flagellar synthesis, cell-proximal portion of basal-body rod (b1073)
flgC	10.91	Flagellar synthesis, cell-proximal portion of basal-body rod (b1074)
flgD	8.92	Flagellar synthesis, initiation of hook assembly (b1075)
flgE	8.67	Flagellar synthesis, hook protein (b1076)
flgF	8.42	Flagellar synthesis, cell-proximal portion of basal-body rod (b1077)
flgG	3.79	Flagellar synthesis, cell-distal portion of basal-body rod (b1078)
flgH	2.37	Flagellar synthesis, basal-body L-ring lipoprotein (b1079)
flgI	2.55	Flagellar synthesis, basal-body P-ring flagellar protein (b1080)
flgJ	2.56	Flagellar synthesis, flagellum-specific muramidase (b1081)
flgKL		
flgK	3.07	Flagellar synthesis, hook-filament junction protein 1 (b1082)
flgL	2.29	Flagellar synthesis; hook-filament junction protein (b1083)
flhBAE		
flhB	1.77	Flagellin export apparatus, substrate specificity protein; determines the order of subunit export (b1880)
flhA	1.51	Flagellar export pore protein, integral membrane protein (b1879)
flhE	1.68	Proton seal during flagellar secretion; periplasmic; bound to flagellar basal body; required for full swarming motility (b1878)
flhDC		
flhD	1.64	Transcriptional activator of flagellar class II operons; forms heterotetramer with FlhC (b1892)
flhC	1.68	Transcriptional activator of flagellar class II operons; CsrA regulon (b1891)
fliAZY		
fliA	6.07	Flagellar synthesis, sigma factor 28 for class III flagellar operons (b1922)
fliZ	2.67	DNA-binding RpoS antagonist (b1921)
fliDST		
fliD	3.73	Flagellar synthesis; filament capping protein; enables filament assembly (b1924)
fliS	1.95	Flagellar chaperone, cytosolic; inhibits premature FliC assembly (c_2340)
fliT	1.50	Flagellar synthesis, predicted chaperone (b1926)
fliFGHIJK		
fliF	3.23	Flagellar synthesis; basal-body M-ring protein (b1938)
fliG	3.46	Flagellar synthesis, component of motor switching and energizing (b1939)
fliH	3.07	Flagellar synthesis; negative regulator of FliI ATPase activity; involved in flagellar assembly and export (b1940)
fliJ	3.12	Flagellin export apparatus soluble chaperone (b1942)
fliK	1.91	Flagellar hook-length control protein (b1943)
fliLMNOPQR		
fliL	7.95	Affects rotational direction of flagella during chemotaxis (b1944)
fliM	7.35	Flagellar synthesis, component of motor switch and energizing (b1945)
fliN	4.59	Flagellar synthesis, component of motor switch and energizing (b1946)

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Table 2 Continued

(a) Polycistronic operons

Gene	Fold change*	Function (KEGG entry)†
fliO	2.38	Flagellar synthesis, flagellin export apparatus, integral membrane protein (b1947)
fliP	1.90	Flagellar synthesis, flagellin export apparatus, integral membrane protein (b1948)
fliQ	2.36	Flagellar synthesis, flagellin export apparatus, integral membrane protein (b1949)
fliR	1.50	Flagellar synthesis, flagellin export apparatus, integral membrane protein (b1950)
guaBA		
guaB	1.75	IMP dehydrogenase (Z3772)
insJK		
insJ	4.29	IS150 transposase A (b3557)
insK	2.30	IS150 transposase B (b3558)
motAB-cheAW		
motA	1.67	Proton conductor component of motor; no effect on switching (b1890)
motB	2.40	Enables flagellar motor rotation, linking torque machinery to cell wall (b1889)
cheA	2.76	Sensory transducer histidine kinase between chemo-signal receptors and CheB and CheY (b1888)
cheW	2.94	Positive regulator of CheA protein activity (b1887)
tar-tap-cheRBYZ		
tar	4.21	Methyl-accepting chemotaxis protein II, aspartate sensor receptor (b1886)
tap	2.01	Methyl-accepting chemotaxis protein IV, peptide sensor receptor (b1885)
cheY	2.52	Response regulator for chemotactic signal transduction, transmits chemoreceptor signals to flagellar motor components; CheA is the cognate sensor protein kinase (b1882)
cheZ	2.01	CheY protein phophatase (Z2935)
purHD		
purH	1.55	Phosphoribosylaminoimidazolecarboxamide formyltransferase; purine synthesis (b4006)
purD	1.50	Phosphoribosylamine-glycine ligase, purine synthesis (b4005)
purMN		
purM	1.56	Phosphoribosyl-aminoimidazole (AIR) synthase (b2499)
purN	1.55	Glycinamide ribonucleotide transformylase (GART) 1, purine synthesis (b2500)
pyrLBI		
pyrB	2.30	Aspartate carbamoyltransferase, catalytic subunit (b4245)
pyrI	1.86	Aspartate carbamoyltransferase, regulatory subunit (b4244)
pyrF-yciH		
pyrF	1.52	Orotidine-5′-phosphate decarboxylase; OMP decarboxylase (b1281)
yciH	1.51	Hypothetical protein (b1282)
upp-uraA		
upp	1.58	Uracil phosphoribosyltransferase (b2498)
yjdA-yjcZ		
yjcZ	1.51	Mutational suppressor of yhjH motility defect, function unknown (b4110)
yjdA	2.49	Mutational suppressor of yhjH motility defect, function unknown (b4109)

(b) Monocistronic operons

Gene	Fold change*	Function (KEGG entry)†
betI	1.51	Probably transcriptional repressor of *bet* genes (b0313)
cspB	1.72	Cold-shock protein; may affect transcription (b1557)
cspF	1.67	Cold-shock protein (b1558)
cspG	1.53	Homologue of *Salmonella* cold-shock protein (b0990)
cspH	1.59	Cold-shock-like protein (b0989)
fliC	3.20	Flagellar synthesis; flagellin structural protein, H-antigen (b1923)
flu	1.62	Antigen 43, phase-variable bipartite outer membrane protein; affects surface properties, piliation, colonial morphology (b2000)
flxA	1.92	Hypothetical protein (b1566)
ompT	1.88	Outer membrane protease VII (b0565)
pyrC	1.53	Dihydroorotase, the third step in pyrimidine biosynthesis (b1062)
pyrD	2.02	Dihydro-orotate dehydrogenase (b0945)
ridA	1.73	Enamine/imine deaminase, required for full IlvE activity and for the dependence of the alternative pyrimidine biosynthesis (APB) pathway of thiamine biosynthesis upon the oxidative pentose phosphate pathway. The *ridA* gene is located immediately downstream of the *pyrLBI* operon and is transcribed in the same direction (b4243)
tsr	1.85	Methyl-accepting chemotaxis protein I; serine chemoreceptor; also senses repellents; belongs to σ^{28} (FliA) flagellar regulon (b4355)
ycgR	4.29	Flagellar velocity braking protein, c-di-GMP-regulated, FlhDC-regulon (b1194)
yecR	1.66	Lipoprotein, function unknown (b1904)
yhjH	1.72	Cyclic-di-GMP phosphodiesterase, FlhDC-regulon; *yhjH* mutants have reduced swimming motility, and overexpression of *yhjH* enhances motility consistent with the model that low cyclic di-GMP favors motility over sessility (b3525)

*Log2 ratios between the corresponding transcript levels of *ΔglgS* and WT cells.

†From http://www.genome.jp/kegg and http://ecogene.org/ecosearch.

include initial attachment of cells to a surface, development of microcolonies, and biofilm maturation, a complex sequence of events involving many different factors that depend considerably on environmental conditions [46].

In *E. coli*, flagellar-derived motility, type 1 fimbriae and the outer membrane Ag43 (antigen 43) adhesin (the product of the *flu* gene) have been implicated in the initial steps of biofilm formation and structural differentiation [33,39,44,46–52]. Because Δ*glgS*

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Table 3 Genes showing significantly reduced transcript levels in *E. coli* BW25113 Δ*glgS* mutants

ORF, open reading frame.

(a) Polycistronic operons

Gene	Fold change*	Function (KEGG entry)†
cysPUWA		
cysP	−1.55	Thiosulfate-binding protein, periplasmatic (c_2959)
cysW	−1.59	Sulfate/thiosulfate ABC transporter membrane permease subunit (b2423)
trpLEDCBA		
trpE	−3.84	Tryptophan synthesis, anthranilate synthase component I (b1264)
trpD	−4.57	Tryptophan synthesis, anthranilate synthase component II, bifunctional; glutamine amidotransferase and phosphoribosyl anthranilate transferase; (b1263)
trpC	−7.18	Tryptophan synthesis, bifunctional: *N*-(5-phosphoribosyl)anthranilate isomerase and indole-3-glycerolphosphate synthase (b1262)
trpB	−10.78	Tryptophan synthase, beta subunit (b1261)
trpA	−7.69	Tryptophan synthase, alpha subunit (b1260)
rpoE-rseABC		
rpoE	−1.53	RNA polymerase, sigma-E factor; heat shock and oxidative stress (b2573)
rseA	−1.52	Sigma-E factor, negative regulatory protein (b2572)
rseB	−1.51	Regulates activity of sigma-E factor (b2571)
yqjCDEK		
yqjD	−1.54	ORF, hypothetical protein (b3099)
yqjE	−1.61	ORF, hypothetical protein (b3099)
yqjK	−1.51	ORF, hypothetical protein (b3100)

(b) Monocistronic operons

Gene	Fold change*	Function (KEGG entry)†
borD	−2.05	Member of PhoPQ regulon; overexpression causes abnormal biofilm architecture; proposed involvement in bacterial virulence (b0557)
cysK	−2.18	Cysteine synthase A (c_2948)
dicC	−1.51	Transcriptional repressor for *dicB* (c_2059)
katG	−1.58	Catalase; hydroperoxidase HPI (b3942)
lpp	−1.91	Murein lipoprotein (b1677)
lrhA	−1.55	Transcriptional repressor of motility master regulator *flhDC* and type 1 fimbriae operons, LysR family (b2289)
lysU	−1.88	Lysine tRNA ligase, heat shock protein (b4129)
mokB	−1.68	ORF, hypothetical protein (b1420)
osmE	−1.69	Osmotically inducible lipoprotein, function unknown (b1739)
osmY	−1.62	Osmotically inducible periplasmic protein, function unknown (b4376)
rmf	−1.80	Ribosome modulation factor (b0953)
sodB	−1.58	Superoxide dismutase (c_2050)
uspB	−1.52	Universal stress protein B (b3494)
wrbA	−1.61	NAD(P)H:quinone oxidoreductase (b1004)
ybaY	−1.55	Novel verified lipoprotein, function unknown (b0453)
ydhR	−1.55	Predicted monooxygenase, function unknown (b1667)
yebV	−1.54	ORF, hypothetical protein (b1836)
ygaM	−1.65	ORF, hypothetical protein (b2672)
ygdI	−1.58	Novel verified lipoprotein, function unknown (b2809)
yiaG	−1.60	ORF, hypothetical protein (b3555)
yjbJ	−1.67	ORF, hypothetical protein (b4045)
yjiY	−2.14	Predicted transporter, function unknown (b4354)

*Log2 ratios between the corresponding transcript levels of Δ*glgS* and WT cells/
†From http://www.genome.jp/kegg and http://ecogene.org/ecosearch.

cells have an increased expression of type 1 fimbriae, Ag43 adhesins and flagellar/motility operons (Table 2), we reasoned that these mutants would have an increased capacity to initiate biofilm formation as compared with the WT cells. To test this hypothesis we compared the ability of WT (BW25113) and Δ*glgS* cells to form a biofilm when grown in polystyrene wells in Kornberg/glucose liquid medium (for details see the Experimental section). We also included in this assay Δ*glgS*Δ*flhC* and Δ*glgS*Δ*fimA* BW25113 double mutants that are impaired in their ability to form flagella and type 1 fimbriae respectively. As shown in Figure 4, Δ*glgS* cells exhibited increased biofilm formation when compared with the WT cells, a phenotype which was largely reverted by ectopic expression of *glgS* in these mutants. Expectedly, the introduction of Δ*flhC*- or Δ*fimA*-null alleles into Δ*glgS* cells reverted their augmented biofilm forming ability, the overall data showing that GlgS exerts a negative effect on biofilm formation.

GlgC-controlled glycogen biosynthesis competes with swarming motility and purine nucleotides metabolic pathway for the same ATP pool in Δ*glgS* cells

Large-scale genetic interaction studies provide the basis for defining gene function and pathway architecture. How GlgS affects glycogen accumulation was investigated by carrying out F-driven conjugation on the basis of large-scale genetic interaction studies. Towards this end Δ*glgS* cells were crossed with the Keio single-gene deletion library, and the double

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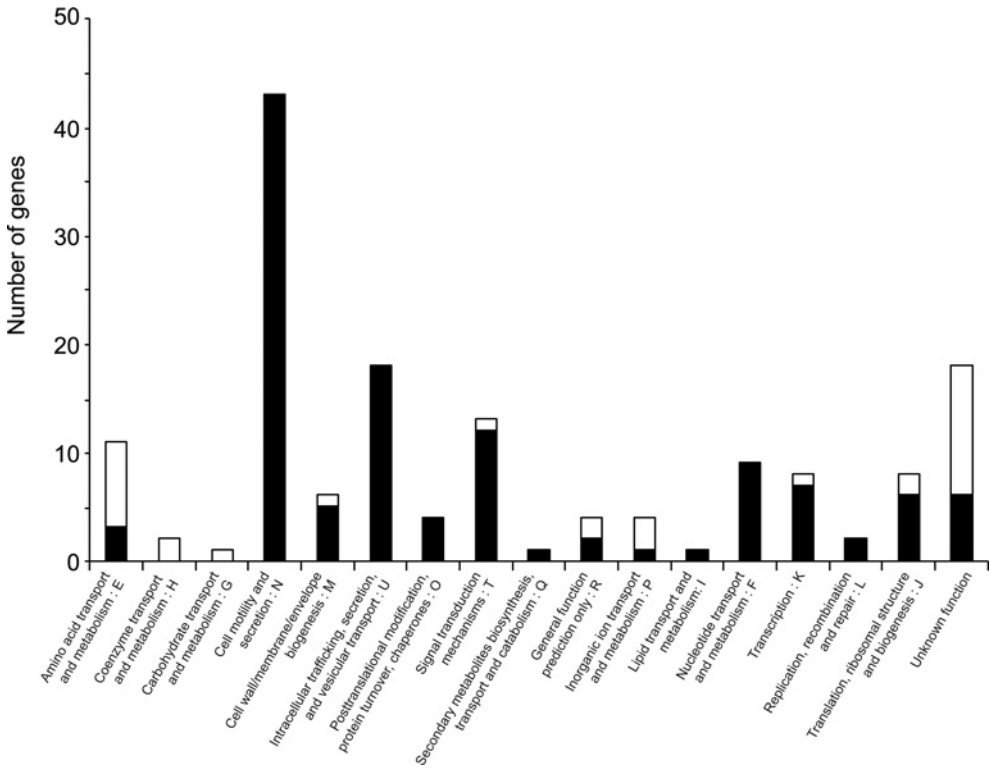

Figure 1 Functional classification of differentially expressed genes in BW25113 Δ*glgS* cells

Solid and open bars represent up- and down-regulated genes respectively in Δ*glgS* cells compared with the WT cells. Genes are classified into COG (clusters of orthologous groups) categories [38].

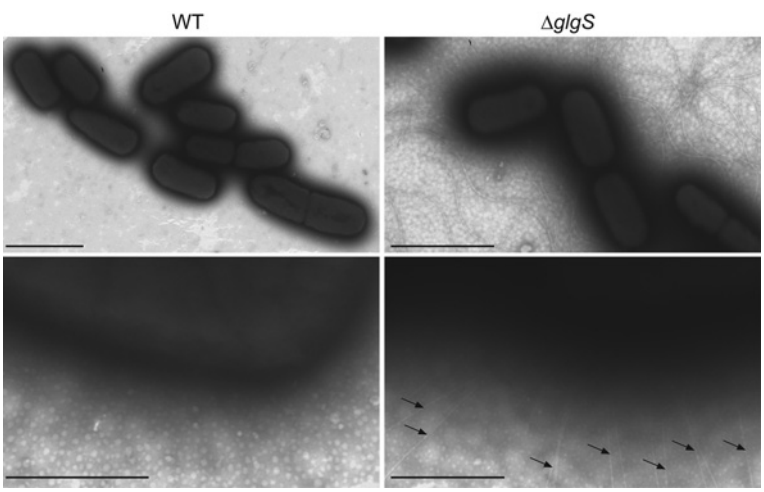

Figure 2 GlgS negatively affects the production of flagella and type 1 fimbriae

Electron microscope images of BW25113 WT and Δ*glgS* cells negatively stained with 2 % phosphotungstic acid. The scale bars in upper and lower panels are 2 and 0.5 μm respectively. Arrows indicate type 1 fimbriae. In the upper panels the Δ*glgS* cells display a hyperflagellated phenotype when compared with the WT cells.

mutants thus obtained were screened for glycogen content using the iodine-staining method (see the Experimental section). On inspecting the mutant library, 32 double mutants accumulated more glycogen than Δ*glgS* cells (Table 4), whereas 36 double mutants accumulated less glycogen than Δ*glgS* cells (Table 5). Consistent with our previous genome-wide screening studies of genes that affect glycogen accumulation [7,12] loss of *pgm* (phosphoglucomutase), *glgA*, *glgC*, *glgB*, *gcvA*, *hfq*, *rpoS*, *prfC* (peptide chain release factor 3), *relA*, *dksA*, *fis*, *trmE*, *trmU* (tRNA 5-methylaminomethyl-2-thiouridylate-methyltransferase) and *ydcQ* (predicted DNA-binding transcriptional regulator) magnified the glycogen-deficient phenotype of Δ*glgS* cells (Table 5). Also consistent with our previous studies, the loss of the genes involved in glycogen breakdown and in the synthesis of amino acids, genes whose deletion causes strongly repressed swarming motility, and genes involved in the *de novo* synthesis of purines that act as major determinants of cell growth, reverted the Δ*glgS* glycogen-deficient phenotype to the WT (Figure 5A and Table 4, and Supplementary Figure S2 at http://www.biochemj.org/bj/452/bj4520559add.htm).

Deletions of the *pur* genes not only resulted in enhanced glycogen content in Δ*glgS* cells, but also reverted the

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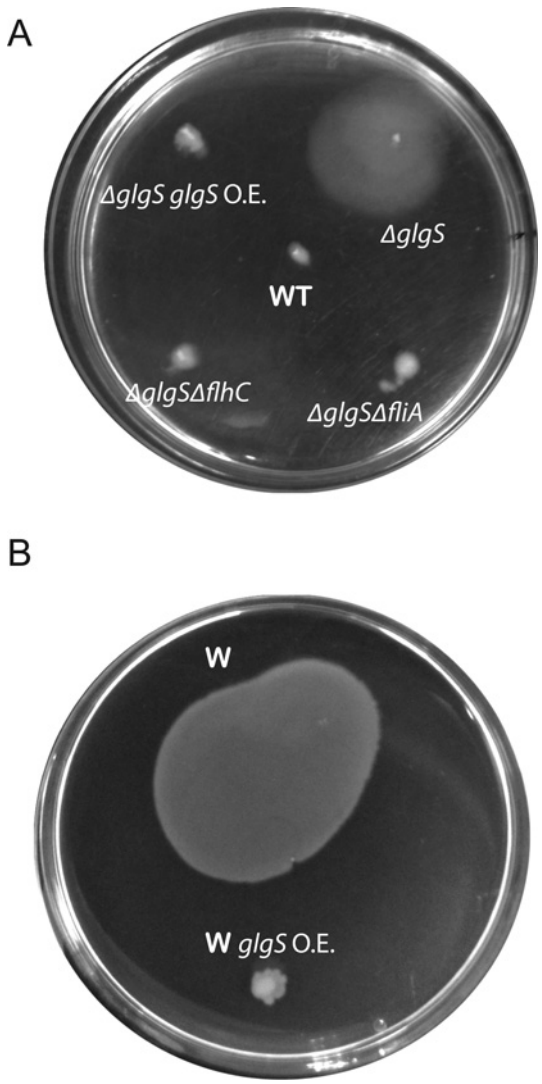

Figure 3 GlgS exerts a negative effect on *E. coli* swarming motility

(**A**) The swarming motility phenotypes of WT (BW25113), Δ*glgS*, Δ*glgS*Δ*flhC*, Δ*glgS*Δ*fliA* and *glgS*-overexpressing (O.E.) Δ*glgS* cells. Note that Δ*glgS* cells display a 'high swarming motility' phenotype, which is complemented by the ectopic expression of *glgS*. As expected, the Δ*glgS*Δ*flhC* and Δ*glgS*Δ*fliA* cells did not swarm, thus confirming that flagella are major determinants of the 'high swarming motility' phenotype of Δ*glgS* cells. (**B**) The swarming motility phenotypes of W cells and *glgS*-overexpressing W cells. The ectopic *glgS* expression exerts a negative effect on the swarming motility of W cells.

hypermotility phenotype of these mutants to the WT (Figure 5B). Because swarming motility and purine biosynthesis are high-ATP consuming processes [53–57] (Supplementary Figure S2), we reasoned that glycogen deficiency in the hypermotile Δ*glgS* cells, and the enhanced glycogen content in the 'low motility' Δ*glgS*Δ*pur* cells (Figure 5A and Table 5), would point to the occurrence of strong competition for the same ATP pool between GlgS-controlled motility and purine nucleotide biosynthesis mechanisms and GlgC-controlled glycogen biosynthesis. Thus, under conditions of high ATP consumption owing to elevated flagellar motility and purine biosynthesis occurring in Δ*glgS* cells, glycogen production will be reduced as a consequence of low GlgC activity resulting from restricted ATP access and increased AMP levels derived from high ATP turnover [7]. Conversely, under conditions of impaired synthesis of purines (such as those occurring in the 'low motility' Δ*glgS*Δ*pur* cells) GlgC will be

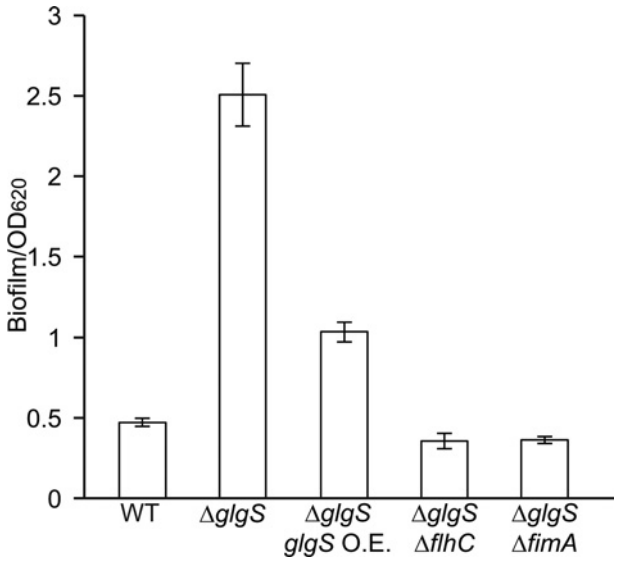

Figure 4 Total biofilm content normalized by bacterial growth (turbidity at 620 nm) in WT, Δ*glgS*, *glgS*-overexpressing (O.E.) Δ*glgS*, Δ*glgS*Δ*flhC* and Δ*glgS*Δ*fimA* cells

Cells were cultured in polystyrene 96-well plates at 28 °C for 48 h in Kornberg medium supplemented with 50 mM glucose and biofilm content was measured as described in the Experimental section. Results are the means ± S.E.M. for five independent experiments.

Table 4 Conjugation-based large-scale identification of gene deletions increasing glycogen accumulation in *E. coli* Δ*glgS* mutants

AICAR, 5-amino-4-imidazolecarboxamide riboside.

Gene	Function in *E. coli* (from http://www.genome.jp)
clpX	ATPase subunit of the two-component ClpXP protease
cpxA	CpxA periplasmic stress sensor histidine kinase
fliA	Transcription factor sigma 28 for class III flagellar operons
flhC	Transcriptional activator of flagellar class II operons; forms heterotetramer with FlhD
flhD	Transcriptional activator of flagellar class II operons; forms heterotetramer with FlhC
fruR	Catabolite repressor-activator Cra
gcvR	Required for repression of *gcv* operon by GcvA
glgP	Glycogen phosphorylase
glgX	Glycogen phosphorylase-limit dextrin α-1,6-glucohydrolase
lysS	Lysine-tRNA ligase
metL	Aspartate kinase/homoserine dehydrogenase
pdxH	Pyridoxine 5′-phosphate oxidase/pyridoxamine 5′-phosphate oxidase
purA	Adenylosuccinate synthetase
purC	Phosphoribosylaminoimidazole-succinocarboxamide synthase
purD	Phosphoribosylamine-glycine ligase
purE	*N*5-carboxyaminoimidazole ribonucleotide mutase
purF	Amidophosphoribosyl transferase
purH	AICAR transformylase/IMP cyclohydrolase
purK	*N*5-carboxyaminoimidazole ribonucleotide synthetase
purL	Phosphoribosylformylglycinamide synthetase
purM	Phosphoribosylformylglycinamide cyclo-ligase
serA	α-Oxoglutarate reductase/D-3-phosphoglycerate dehydrogenase
serB	Phosphoserine phosphatase
serC	Phosphohydroxythreonine aminotransferase/3-phosphoserine aminotransferase
wzxE	O-antigen translocase; involved in the cross-membrane translocation of the UDP-linked ECA trisaccharide repeat unit of enterobacterial common antigen ECA(CYC)
ycfR	Protein involved in stress resistance and biofilm formation
ydaF	Rac prophage; predicted protein
yehQ	Predicted protein
yfdG	Bactoprenol-linked glucose translocase
ynfB	Predicted protein
yqiJ	Inner membrane protein, function unknown
yqiK	PHB family inner membrane protein, function unknown

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Table 5 Conjugation-based large-scale identification of gene deletions further reducing glycogen accumulation in *E. coli* Δ*glgS* mutants

Gene	Function in *E. coli* (from http://www.genome.jp)
aspC	Aspartate aminotransferase
dam	DNA adenine methyltransferase
ddlB	D-alanine: D-alanine ligase
dksA	RNA-polymerase-binding protein modulating ppGpp and iNTP regulation
dsrA	Sulfite reductase, dissimilatory-type alpha subunit
essQ	Qin prophage; predicted S lysis protein
fis	Transcriptional activator for rRNA operons
gcvA	Transcriptional repressor for the *gcv* operon
gidA	Uridine 5-carboxymethylaminomethyl modification enzyme
glgA	Glycogen synthase
glgB	1,4-alpha-glucan branching enzyme
glgC	Glucose-1-phosphate adenylyltransferase
glnP	Glutamine transport system permease
gnd	6-Phosphogluconate dehydrogenase
hda	Regulator of DnaA that prevents premature reinitiation of DNA replication
hdhA	7-α-Hydroxysteroid dehydrogenase
hfq	Host factor-I protein
mhpT	3-Hydroxyphenylpropionic acid transporter
miaA	tRNA dimethylallyltransferase
minC	Inhibition of FtsZ ring polymerization
moaE	Molybdopterin synthase catalytic subunit
pgm	Phosphoglucomutase
prfC	Peptide chain release factor RF-3
prmB	Putative adenine-specific DNA-methyltransferase
puuP	Putrescine importer
relA	(p)ppGpp synthase
rpoS	General stress response sigma factor
sufC	Fe–S cluster assembly ATP-binding protein
trmE	tRNA modification GTPase
trmU	tRNA-specific 2-thiouridylase
ydaT	Required for swarming phenotype, function unknown
ydcQ	Mutational suppressor of null *rpoE* lethality
yeaD	Glucose-6-phosphate 1-epimerase
ymfT	Cro-like repressor
ymgA	Connector protein for RcsB regulation of biofilm formation
ynjA	Function unknown

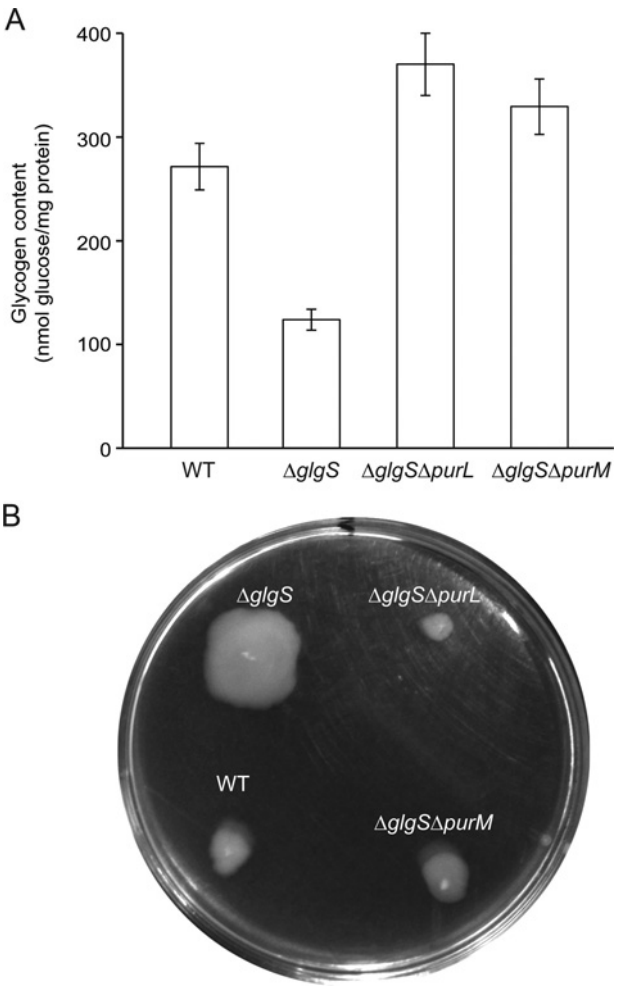

Figure 5 Impairment in the *de novo* synthesis of purines results in enhanced glycogen content and reverts the hypermotility phenotype of Δ*glgS* cells

Glycogen content (**A**) and swarming motility (**B**) of WT (BW25113), Δ*glgS*, Δ*glgS*Δ*purM* and Δ*glgS*Δ*purL* cells. Results are the means ± S.E.M. for three independent experiments.

active and compete for surplus ATP with the swarming motility mechanisms therefore promoting net glycogen accumulation.

Whether GlgS-controlled swarming motility and GlgC-controlled glycogen production compete for the same ATP pools was examined by analysing the swarming motility in the glycogen-less Δ*glgC* cells. We also analysed the effect of *glgC* overexpression on glycogen content and swarming motility in the hypermotile BW25113 Δ*glgS* cells. Furthermore, we compared the glycogen content between the hypermotile Δ*glgS* cells and the low motility Δ*glgS*Δ*flhC* and Δ*glgS*Δ*fliA* cells. As shown in Figure 6, these analyses revealed that the glycogen-less Δ*glgC* cells showed exceedingly higher swarming motility than the WT cells. In turn, both the reduced glycogen content and increased swarming motility of Δ*glgS* mutants could be reverted to the WT by *glgC* overexpression (Figure 6). Furthermore, the 'low motility' Δ*glgS*Δ*flhC* and Δ*glgS*Δ*fliA* double mutants (Figure 3A) accumulated higher glycogen than Δ*glgS* cells (Figure 6A). The overall data thus indicate that the mechanisms involved in glycogen biosynthesis and swarming motility/growth compete for the same ATP pools; the low glycogen phenotype of the hypermotile Δ*glgS* cells being ascribed, at least in part, to low ATP-consuming GlgC activity as a consequence of (i) high ATP consumption owing to increased flagellar motility and growth, and/or (ii) high intracellular AMP levels. Alternatively, it is possible that glycogen deficiency may indirectly promote the high motility phenotype of Δ*glgS* and Δ*glgC* cells. To investigate this hypothesis we analysed the motility of Δ*glgA* cells of the Keio collection, which display a glycogen-less phenotype owing to the absence of glycogen synthase [7] (Figure 6A), but still expresses ATP-consuming GlgC. As shown in Figure 6(B) Δ*glgA* cells displayed a nearly WT swarming motility phenotype, ruling out the possibility that the high swarming motility of Δ*glgC* and Δ*glgS* cells could be ascribed to glycogen deficiency.

GlgC-controlled glycogen biosynthesis competes with biofilm polysaccharide biosynthetic pathways for the same G1P pools in Δ*glgS* cells

EPSs are major components of most biofilm matrices that can either remain associated with the cell wall to form capsule layers or be released into the milieu as an extracellular slime. Synthesis of EPSs, such as colanic acid, depends on the metabolic conversion of G1P and UTP into UDPG (UDP-glucose) by means of GalU (UTP–glucose-1-phosphate uridylyltransferase; Supplementary Figure S3 at http://www.biochemj.org/bj/452/bj4520559add.htm). As shown in Figure 7(A), deletions of *pur* genes limiting purine biosynthesis not only reverted the hypermotility and 'low glycogen' phenotypes of Δ*glgS* cells

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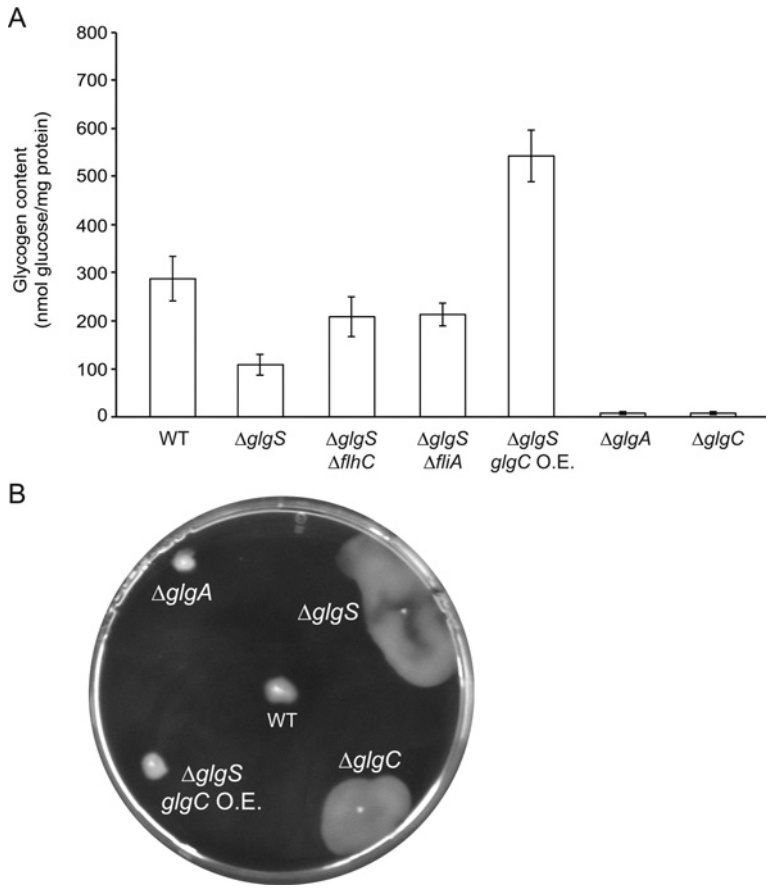

Figure 6 GlgC-controlled glycogen biosynthesis and swarming motility compete for the same ATP pool in Δ*glgS* cells

(**A**) Glycogen content in WT (BW25113), Δ*glgS*, Δ*glgS*Δ*flhC*, Δ*glgS*Δ*fliA*, *glgC*-overexpressing (O.E.) Δ*glgS*, Δ*glgA* and Δ*glgC* cells. (**B**) Swarming motility in WT, Δ*glgS*, Δ*glgA*, Δ*glgC* and *glgC*-overexpressing Δ*glgS* cells. Results are the means ± S.E.M. for three independent experiments.

(Figure 5), but also their 'high biofilm' phenotype. Because both GalU-dependent EPS synthesis and GlgC-dependent glycogen synthesis are G1P-dependent processes, we reasoned that the glycogen-deficient phenotype of the 'high-biofilm' Δ*glgS* cells and the enhanced glycogen content of the 'low-biofilm' Δ*glgS*Δ*pur* cells (Figure 5A) would point to the occurrence in Δ*glgS* cells of strong competition for the same G1P pools between GalU-dependent EPS synthetic mechanisms and GlgC-dependent glycogen biosynthesis. Thus, under conditions of high ATP-consuming flagellar motility and a high content of AMP derived from high ATP turnover occurring in the 'high biofilm' Δ*glgS* cells, glycogen production will be reduced as a consequence of low GlgC activity and surplus G1P will be then available for an increased synthesis of biofilm EPSs. To test this hypothesis we measured the biofilm content in *glgC*-overexpressing Δ*glgS* cells and in the glycogen-less Δ*glgC* cells. We also measured the colanic acid content in the WT, Δ*glgS* and Δ*glgC* cells, as well as in Δ*glgS* and Δ*glgC* cells ectopically expressing *glgC*. As negative controls we used Δ*galU* and Δ*wzc* cells impaired in colanic acid and biofilm production. It is noteworthy that these analyses revealed that the glycogen-deficient Δ*glgC* and Δ*glgS* mutants accumulate a high content of biofilm and colanic acid, phenotypes that were reverted to the WT by the ectopic expression of *glgC* and by introduction of Δ*galU* or Δ*wzc* alleles (Figure 7). The overall data would thus indicate that the pathways involved in the synthesis of glycogen and biofilm EPSs compete for the same G1P pools. In this proposal, the 'low glycogen' and 'high biofilm' phenotypes of Δ*glgS* cells could be ascribed, at least in part, to reduced GlgC activity owing to a high AMP intracellular content and/or restriction to ATP access (see above), the surplus G1P being diverted towards EPS biosynthesis.

Additional remarks and proposal of an integrated model for the GlgS-mediated regulation of synthesis of flagella, type 1 fimbriation, and the production of glycogen and biofilm exopolysaccharides in *E. coli*

The results of the present study indicate that GlgS, previously thought to represent a glycogen synthesis control protein in *E. coli*, is a functional regulator of the formation of the surface organelles and pathways responsible for cell motility and growth, chemotaxis, adhesion, and biofilm formation. GlgS loss resulted not only in an increased expression of flagella-related genes, but also in increased production and relocation of the proteins required for the complete assembly and normal function of flagella, which are processes tightly controlled in a cascade fashion with a hierarchy to meet a cell's immediate needs for motility and to prevent undesirable energy costs [55]. Because GlgS is a major determinant of *E. coli* surface composition, and because its effect on glycogen metabolism appears to be only indirect, we propose to rename this protein as ScoR, for surface composition regulator.

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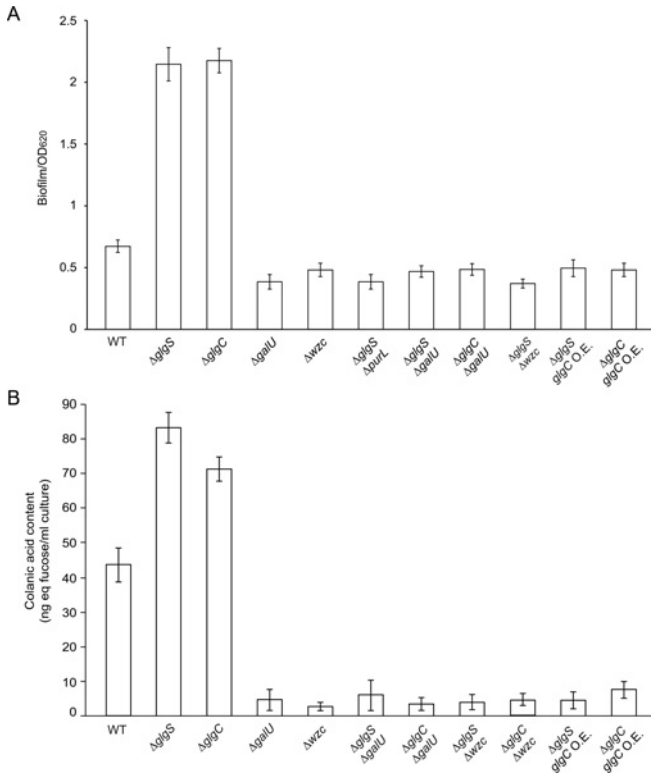

Figure 7 GlgC-controlled glycogen biosynthesis competes with biofilm polysaccharide biosynthetic pathways for the same G1P pools in Δ*glgS* cells

(**A**) Biofilm content normalized by bacterial growth (turbidity at 620 nm) in WT (BW25113), Δ*glgS*, Δ*glgC*, Δ*galU*, Δ*wzc*, Δ*glgS*Δ*purL*, Δ*glgS*Δ*galU*, Δ*glgC*Δ*galU*, Δ*glgS*Δ*wzc*, *glgC*-overexpressing (O.E.) Δ*glgS* and *glgC*-overexpressing Δ*glgC* cells. (**B**) Colanic acid in WT, Δ*glgS*, Δ*glgC*, Δ*galU*, Δ*wzc*, Δ*glgS*Δ*galU*, Δ*glgC*Δ*galU*, Δ*glgS*Δ*wzc*, Δ*glgC*Δ*wzc*, *glgC*-overexpressing Δ*glgS* and *glgC*-overexpressing Δ*glgC* cells. Results are the means ± S.E.M. for three independent experiments. Note that the 'high colanic acid content' phenotype of Δ*glgC* and Δ*glgS* cells is reverted to the WT by the incorporation of Δ*galU* and Δ*wzc* alleles, providing evidence that the 'high biofilm content' and 'high colanic acid content' phenotypes of glycogen-deficient Δ*glgS* cells and glycogen-less Δ*glgC* cells are ascribed, at least in part, to GalU-mediated conversion of surplus G1P into EPSs.

Figure 8 illustrates a suggested integrated model for the GlgS (ScoR)-controlled regulation of synthesis of flagella, type 1 fimbriae, purine, pyrimidine, biofilm EPSs and glycogen wherein the general stress regulator RpoS acts as one of the major determinants of *glgS (scoR)* expression. RpoS levels and activity are determined, in part, by the cellular levels of the RelA and SpoT products (p)ppGpp [58]. Accumulation of (p)ppGpp in *E. coli* cells facing nutritional and other environmental stress situations leads to the restructuring of global gene expression patterns and cell regulatory networks (often referred to as the 'stringent response') aimed at rapidly adapting cell metabolism to newly deteriorating conditions, the protection of cellular structures and long-term survival [59–61]. In *E. coli*, (p)ppGpp exerts a positive effect on glycogen accumulation since it: (i) transcriptionally up-regulates the expression of both the *glgBXCAP* operon [7,10,12,19] and the small non-coding RNA *csrC* [19,62], which in turn inactivates the glycogen biosynthetic post-transcriptional repressor CsrA [63]; and (ii) potently inhibits PurA [64], which catalyses the first committed step in the *de novo* biosynthesis of the main GlgC inhibitor AMP. In contrast, (p)ppGpp exerts a negative effect on motility and adhesion to surfaces since it promotes the RpoS-mediated repression of type 1 fimbriae [65,66] and represses the expression of flagellar and *de novo* pyrimidine synthesis genes [18,19,67], the latter acting as major determinants for EPSs production [68] and growth. Thus, according to the suggested integrated metabolic model illustrated in Figure 8, when cells initiate growth and nutrients are in excess, *glgBXCAP* and *scoR (glgS)* expression will be reduced as a consequence of the low (p)ppGpp and RpoS levels and the high levels of active CsrA, a situation which: (i) disfavours glycogen accumulation; and (ii) allows the expression of flagellar and type 1 fimbriae operons and of operons involved in the synthesis of purines and pyrimidines, all factors required for increased adhesion of cells to surfaces, motility and growth. Conversely, when growing cells start to face nutrient limitation, the augmentation of (p)ppGpp and RpoS levels and/or sRNA *csrC*-mediated inactivation of CsrA will enhance the expression of both *glgBXCAP* (thus resulting in enhanced glycogen accumulation when a carbon source is present) and *scoR (glgS)* (resulting in a general down-regulation of the production of surface organelles involved in motility and adhesion, and in the activity of nucleotide biosynthetic pathways therefore restricting growth). We must emphasize that ScoR (GlgS)-mediated down-regulation of flagellar functions under stringent conditions makes physiological sense in that flagella production and motility impose a high energy burden on the cell [53], and limiting their expression to favour the production of reserve glycogen would be advantageous for cell survival.

In WT *E. coli* cells flagella and glycogen production are non-concomitant processes, the former mainly occurring during the early stages of exponential cell growth and the latter mainly occurring during the transition from the exponential growth to the stationary phase [7,69]. Such control is seemingly absent in Δ*scoR (glgS)* cells, which exhibit a deregulated and constitutive expression of high energy-demanding flagellar motility and G1P-consuming EPS biosynthetic processes competing with GlgC for the same ATP and G1P pools respectively during the stationary phase. Consequently, glycogen production in Δ*scoR (glgS)* cells will be reduced when compared with the WT cells, the surplus ATP and G1P being diverted towards flagellar propulsion and biofilm EPS production respectively.

The molecular mechanism(s) beneath the action of ScoR (GlgS) are under investigation in our laboratory. We must emphasize that although previous ScoR (GlgS) structural analysis indicated that this protein has the ability to interact with other proteins [15], we systematically failed to identify interactions between ScoR (GlgS) and any protein encoded by the *glgBXCAP* operon (results not shown). It is thus tempting to speculate that the reduced glycogen content and global transcriptional changes observed in Δ*scoR (glgS)* cells reflect ScoR (GlgS) interactions with key transcription factors regulating the expression of genes such as those involved in type 1 fimbriation, synthesis of flagella, and purine and pyrimidine nucleotide biosynthesis. Alternatively, ScoR (GlgS) could also act by regulating the stability of transcripts of key transcriptional regulators. In this context it is worth mentioning that our transcriptome analysis indicated that *lrhA* transcripts are down-regulated in BW25113 Δ*scoR* (*glgS*) mutants (Table 3 and Supplementary Figure S1). Similar to ScoR (GlgS), LrhA represses the expression of type 1 fimbrial adhesins and flagellar motility and chemotaxis genes [34,70]. Thus, by directly or indirectly controlling the transcript levels of *lrhA*, ScoR (GlgS) could induce profound effects on *E. coli* motility, initial surface attachment and subsequent biofilm development. Our analysis also revealed that the transcript levels of genes involved in both *de novo* and salvage purine and pyrimidine synthesis pathways are higher in Δ*scoR (glgS)* cells than in the WT cells (Table 2). Most notably, all of these genes form part of the PurR regulon, being negatively regulated by the PurR repressor under conditions of excess availability of purine nucleotides [57]. Thus, by directly

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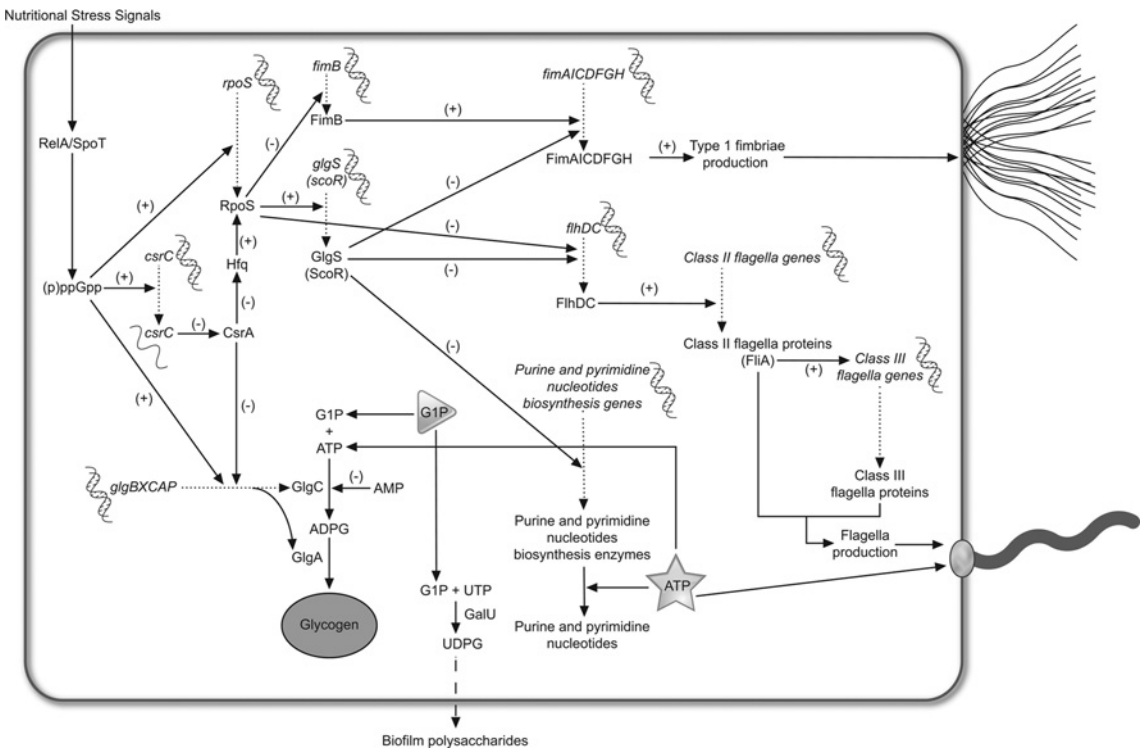

Figure 8 Suggested integrated scheme for GlgS (ScoR)-controlled synthesis of flagella, type 1 fimbriae, biofilm polysaccharides and glycogen

According to this model, *glgS* (*scoR*) expression is mainly determined by RpoS, whose levels are in turn determined by (p)ppGpp produced by RelA and SpoT when *E. coli* cells face nutritional and other environmental stress situations. In *E. coli*, (p)ppGpp exerts a positive effect on glycogen accumulation since it: (i) transcriptionally up-regulates the expression of both the *glgBXCAP* operon and the small non-coding RNA *csrC*, which in turn inactivates the glycogen biosynthetic post-transcriptional repressor CsrA; and (ii) potently inhibits PurA, which catalyses the first committed step in *de novo* biosynthesis of the main GlgC inhibitor AMP. (p)ppGpp also exerts a negative effect on the production of flagella and EPSs since it represses the expression of flagellar genes and *de novo* pyrimidine synthesis genes acting as major determinants for EPSs production. According to this suggested integrated metabolic model, when nutrients are in excess, *glgBXCAP* and *glgS (scoR)* expression will be reduced as a consequence of the reduced (p)ppGpp and RpoS levels and the high levels of active CsrA, a situation which will: (i) disfavour glycogen accumulation; and (ii) allow the expression of FlhDC-regulated flagellar operons, type 1 fimbriae genes and of operons involved in the synthesis of purine and pyrimidine nucleotides necessary for growth. Conversely, under stringent conditions, augmentation of (p)ppGpp and RpoS levels, and/or small non-coding RNA *csrC*-mediated inactivation of CsrA will enhance the expression of both *glgBXCAP* (thus resulting in enhanced glycogen accumulation when a carbon source is present) and *glgS (scoR)*, which in turn will down-regulate the expression of operons involved in the synthesis of flagella, type1 fimbriae, and purine and pyrimidine nucleotides. According to this suggested model, the lack of GlgS (ScoR) will promote the constitutive production of flagella and EPSs that will compete with GlgC for the same ATP and G1P pools respectively, thus resulting in 'glycogen-deficient' and hypermotile cells.

or indirectly controlling PurR functions, ScoR (GlgS) may also help by regulating the use of available cellular resources when nutrients become scarce. Finally, our RNA array analyses revealed that the transcript levels of indole biosynthetic genes such as *trp* (tryptophan synthase) *A*, *trpB*, *trpC*, *trpD and trpE* in Δ*scoR (glgS)* cells are lower than in the WT cells (Table 3 and Supplementary Figure S1). Similar to ScoR (GlgS), indole restricts biofilm formation probably as a consequence of its negative effect on the expression of type 1 fimbriae and flagellar genes [71,72], and thus, by controlling indol metabolism, ScoR (GlgS) could induce profound effects on *E. coli* motility, initial surface attachment and the subsequent biofilm development. Needless to say, further efforts will be necessary to investigate the possible occurrence of complex relationships that link ScoR (GlgS), indole, PurR and LrhA in the regulation of *E. coli* motility, surface attachment and biofilm formation.

Motility and adhesion organelles are important bacterial virulence factors required for the initial steps of biofilm formation and are the main cause of severe problems in medical, environmental and industrial settings [33,44,51,73–77]. The findings of the present study identifying *E. coli* ScoR (GlgS) as a key negative regulator of their synthesis may thus point to a valuable target for the development of antimicrobial agents aimed to control biofilm development and persistence mechanisms on recalcitrant pathogens.

AUTHOR CONTRIBUTION

Mehdi Rahimpour, Manuel Montero, Goizeder Almagro, Ángel Sevilla, Manuel Cánovas, Francisco Muñoz, Edurne Baroja-Fernández, Abdellatif Bahaji, Gustavo Eydallin, Hitomi Dose and Rikiya Takeuchi performed the experiments. Mehdi Rahimpour, Manuel Montero, Goizeder Almagro, Alejandro Viale, Hirotada Mori and Javier Pozueta-Romero planned the experiments and analysed the data. Mehdi Rahimpour, Manuel Montero, Goizeder Almagro, Alejandro Viale and Javier Pozueta-Romero wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

GlgS, described previously as a glycogen synthesis control protein, negatively regulates motility and biofilm formation in *Escherichia coli*

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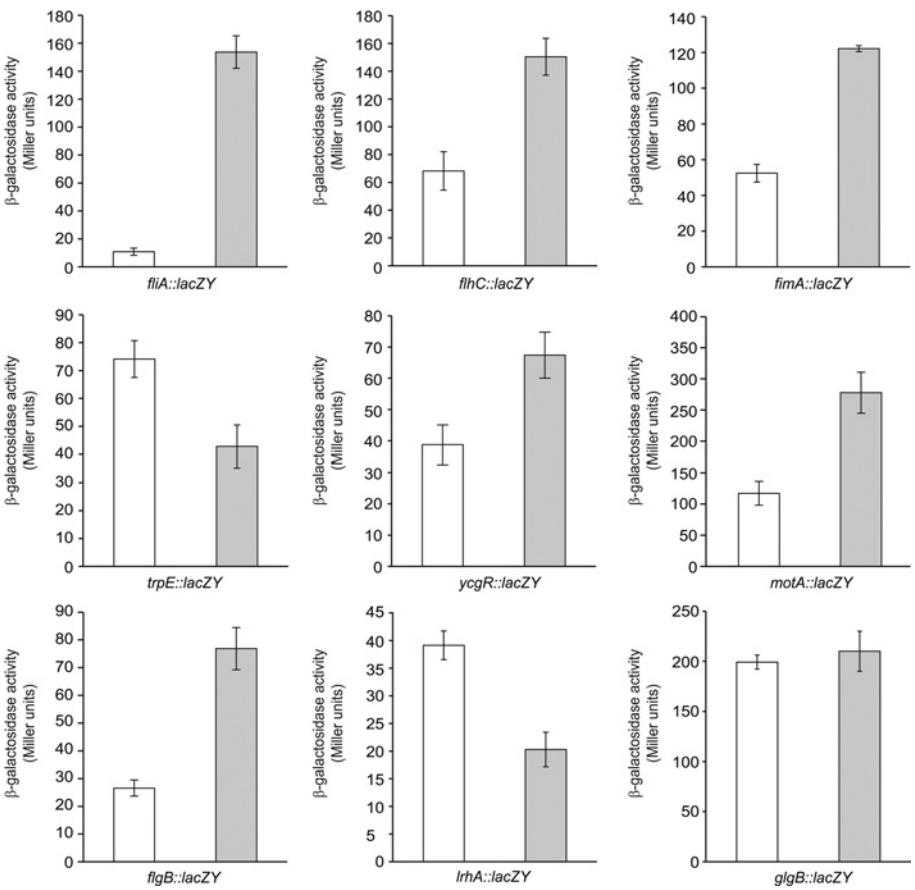

Figure S1 β-Galactosidase activities of WT cells (white bars) and Δ*glgS* cells (grey bars) expressing the indicated *lacZY* transcriptional fusions

Cells were cultured in liquid Kornberg/glucose medium and harvested at the onset of the stationary phase for β-galactosidase activity measurements. Results are the means$\pm$S.E.M. for three independent experiments. For further details see the Experimental section of the main text.

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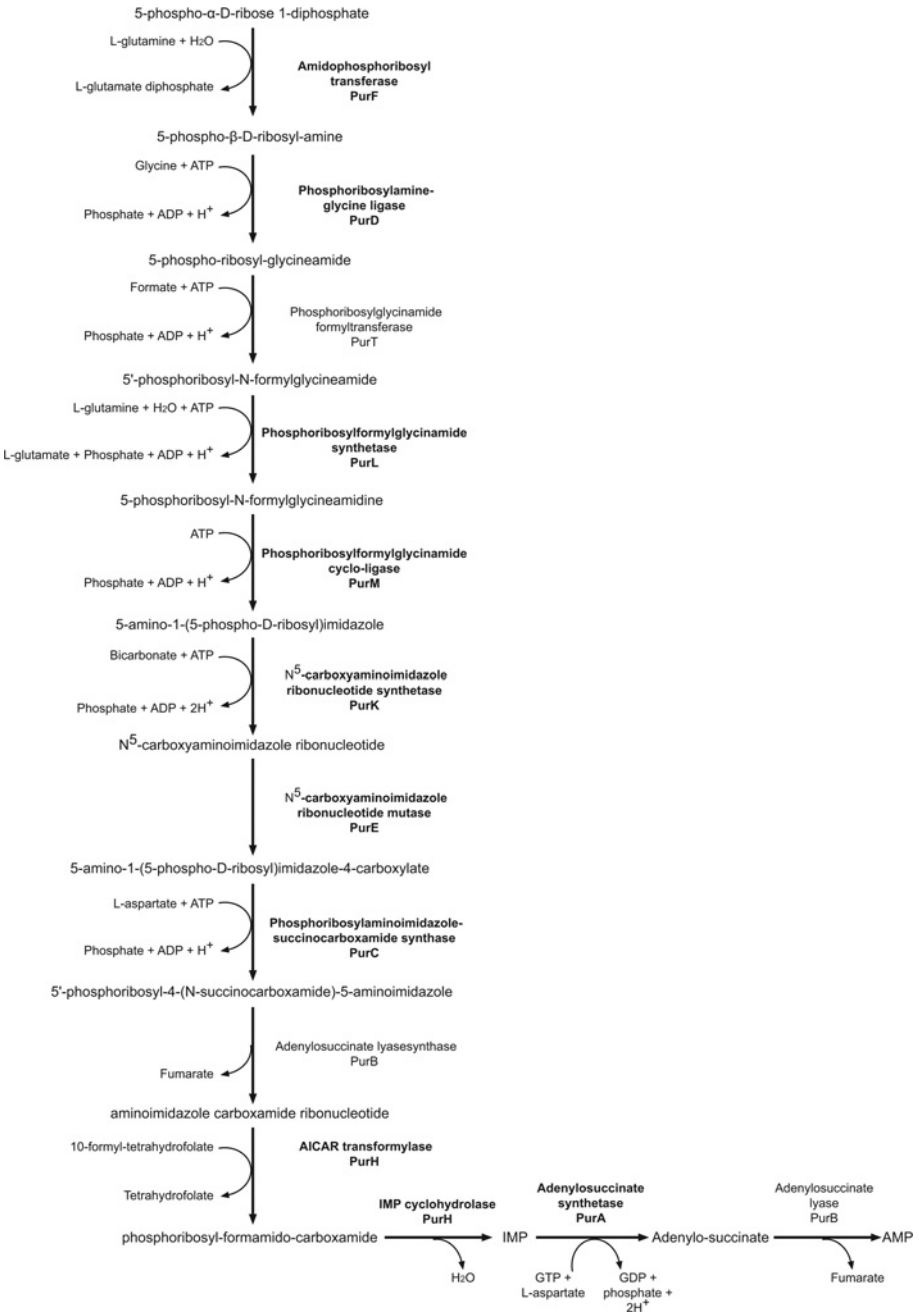

Figure S2 Metabolic pathway for *de novo* synthesis of purines

The enzymes whose down-regulation promotes glycogen accumulation in Δ*glgS* cells are indicated in bold.

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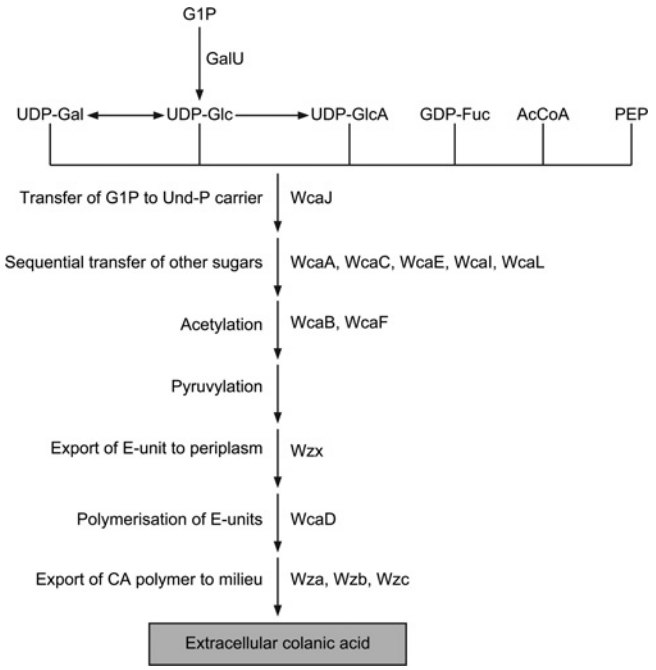

Figure S3 Metabolic conversion of G1P into colanic acid (CA) in *E. coli* cells

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