Streptomyces tsukubaensis as a new model for carbon repression: transcriptomic response to tacrolimus repressing carbon sources

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ABSTRACT

In this work, we identified glucose and glycerol as tacrolimus repressing carbon sources in the important species *Streptomyces tsukubaensis*. A genome-wide analysis of the transcriptomic response to glucose and glycerol additions was performed using microarray technology. The transcriptional time series obtained allowed us to compare the transcriptomic profiling of *S. tsukubaensis* growing under tacrolimus producing and non-producing conditions. The analysis revealed important and different metabolic changes after the additions and a lack of transcriptional activation of the *fkb* cluster. In addition, we detected important differences in the transcriptional response to glucose between *S. tsukubaensis* and the model species *Streptomyces coelicolor*. A number of genes encoding key players of morphological and biochemical differentiation were strongly and permanently downregulated by the carbon sources. Finally, we identified several genes showing transcriptional profiles highly correlated to that of the tacrolimus biosynthetic pathway regulator FkbN that might be potential candidates for the improvement of tacrolimus production.

INTRODUCTION

Strains of the gram-positive, soil-dwelling bacterial genus *Streptomyces* stand out for their ability to produce a wide range of secondary metabolites with biological activity. In fact, more than a half of the antibiotics from microbial origin used in clinics are produced by this genus (Hopwood 2007). *Streptomyces tsukubaensis* (Kino et al. 1987a; 1987b) is an important industrial species which produces tacrolimus (or FK506), a 23-membered macrolide showing immunosuppressant activity that is widely used in the prevention of graft rejection and in the treatment of skin diseases. Despite of its clinical relevance and the generation of important benefits for the pharmaceutical market, low production levels are achieved by industrial strains (Barreiro and Martínez-Castro 2014). Improvement of FK506 production has been obtained through culture media optimization and genetic engineering of the strains. Nevertheless, the identification of transcriptional regulators that might be involved in the regulation of its biosynthesis is of high interest to achieve further improvements.

The presence of carbon sources in the culture media that are rapidly assimilated blocks or reduces the production of secondary metabolites and such regulation can take place at the enzymatic and/or at the transcriptional level (reviewed in Ruiz et al. 2010). This phenomenon resembles carbon catabolite repression (CCR; Magasanik 1961), which prevents the use of alternative carbon sources in the presence of "preferred" ones (usually glucose). As it can be deduced, CCR is an important barrier for the production of bioactive compounds, since preferred carbon sources that would allow a faster growth hamper secondary metabolite production. Thus, avoiding or reducing CCR is an important strategy to improve secondary metabolite production and, for this purpose, understanding its regulation is highly necessary. Despite of its importance, the molecular mechanisms that govern CCR in the genus *Streptomyces* are still not completely elucidated. A key player in *Streptomyces* CCR is the glycolytic enzyme glucose kinase (Glk), which is proposed to interact with transcriptional regulators in order to exert its regulatory role (Angell et al. 1992). Nevertheless, Glk is not the sole responsible for *Streptomyces* CCR; other players such as SCO2127 or Rok7b7 are involved (Angell et al. 1992; Gubbens et al. 2012).

Since "omic" approaches represent a useful tool to study regulatory networks, the aim of this work was i) to identify FK506 repressing carbon sources in *S. tsukubaensis* and ii) to study their effect on the whole transcriptome and establish a comparison between the transcriptional behaviour of this strain under FK506 producing and non-producing conditions. By this mean we aimed to identify key regulators that might be involved in FK506 production and/or in the mechanisms governing CCR. Transcriptomics have been applied recently to the study of *S. coelicolor* CCR in a one-point experimental design corresponding to the exponential growth phase (Romero-Rodríguez et al. 2016a; 2016b). In this work, we performed a 10-point transcriptional time series comprising all the growth phases. Such design enables the comparison not only between producing and non-producing conditions but also between primary and secondary metabolism. Here we describe the main transcriptional changes observed after glucose and glycerol additions and present new candidates for the improvement of FK506 production and the study of key *Streptomyces* biology aspects.

MATHERIALS AND METHODS

Bacterial strains and growth conditions

Streptomyces tsukubaensis (Kino et al. 1987a) was grown at 28 ºC on ISP4 (Difco™, BD, NJ, USA) medium for spore preparation. For FK506 production studies, 10^9 spores of *S. tsukubaensis* were inoculated into 0.5-l flasks containing 100 ml of MGm-2.5 media (Martínez-Castro et al. 2013) and incubated at 28 ºC and 220 rpm. Carbon sources added to the cultures, such as glycerol, mannitol (both form Prolabo-VWR, Radnor, PA, USA), D-fructose (Merck, Darmstadt, Germany), maltose monohydrate (SAFC-Sigma, Madison, WI, USA), xylose, D-glucose monohydrate (both from Sigma-Aldrich, St. Luis, MI, USA), sucrose (NormaPur--VWR, Radnor, PA, USA) and lactose monohydrate (Rectapur--VWR, Radnor, PA, USA), were dissolved in hot Milli-Q water (65 ºC) and sterilized at 120 ºC for 15 minutes. The final concentrations in the culture media are indicated in the corresponding section.

The FK506-sensitive strain *Saccharomyces cerevisiae* TB23 (Breuder et al. 1994) was cultured in YPD media (Lodder, 1970) at 28 ºC and 250 rpm.

Growth measurement, FK506 and phosphate determination

For growth measurement and phosphate determination, 2-ml culture samples were harvested and centrifuged. The supernatant was collected for inorganic phosphate determination using the malachite green assay (Lanzzeta et al. 1979). The pellet was washed twice with Milli-Q® water and dried at 80 ºC for 48 hours for growth determination.

For FK506 extraction, 1-ml culture samples were mixed with an equal volume of methanol (HPLC grade) in 10-ml tubes. The mixtures were shaken in a horizontal position for one hour at 140 rpm and centrifuged. The supernatants were collected and FK506 concentration was measured with an Agilent HPLC equipped with a Zorbax SB C18 column (4.6x150 mm, 3.5 µm) following the indications from Salehi-Najafabadi and coworkers (2014). Standards of pure FK506 (Antibióticos de León SLU, Spain) and ascomycin (Sigma-Aldrich, St. Luis, MI, USA) were used as controls.

During the screening for repressing carbon sources, antifungal activity in the extracts was detected by bioassay against *S. cerevisiae* TB23 (Breuder et al. 1994) as indicated by Ordóñez-Robles and coworkers (2016).

RNA extraction and purification, labelling and hybridization

All the procedures related to the extraction and purification of RNA, the synthesis of labeled cDNA and the conditions used for microarray hybridization were performed as previously described (Ordóñez-Robles et al. 2016). Samples for RNA extraction were taken at 70 h (immediately before the additions), 70.7 h, 72 h, 76 h, 80 h, 89 h, 92 h, 100 h, 124 h and 148 h.

Microarray design and data analysis

The custom microarrays used in this work were manufactured by Agilent Technologies (Santa Clara, CA, USA) in the 8×15K format. The expression probes (45- to 60-mer) were designed using the online tool eArray from Agilent. In addition, tiling probes covering the coding strand of the FK506 biosynthetic cluster (*fkb*) were designed using the chipD program (Dufour et al. 2010).

The limma package v3.20 (Smyth 2004) was used for normalization of the signal intensities and also for statistical analyses following the indications in Ordóñez-Robles and coworkers (2016). After normalization we obtained a final M_g value ($\log_2$ transcription value), which is an approximate measure of the abundance of the transcripts of a particular gene with respect to its genomic copies (Mehra et al. 2006; Sidders et al. 2007). To find differentially expressed genes limma calculated the M_c values, which represent the $\log_2$-fold change between two experimental conditions (i. e. differences between selected M_g values). Limma also provided the adjusted *p*-values (named p_{FDR}) to control the false discovery rate (Benjamini and Hochberg 1995). The maSigPro software (Conesa et al. 2006), from the Bioconductor 3.2 package, was used to find genes showing different transcription profiles between experimental conditions during the five first time points of the series. To detect transcriptional profiles similar to that of the transcriptional regulator coding gene *fkbN*, we analyzed Pearson correlation coefficients.

Quantitative reverse transcription PCR (RT-qPCR)

To validate the microarray results by RT-qPCR, we used the primer pairs listed in Table S1 and the procedures indicated by Ordóñez-Robles and coworkers (2016). cDNA originated from the RNA samples was used to measure transcript levels of *pfkA1, pfkA2, pfkA3, amtB, hrdA, gltD, fkbN, glpX, crp* and *phoP*. For normalizing assays, *metF* and *gyrB* genes were chosen since their M_g levels were among the most constant throughout the time series. A high correlation (R^2 = 0.78) between microarray-derived and RT-qPCR transcriptional ratios validated the results (see Fig S1).

RESULTS

Experimental setup

Identification of FK506-repressing carbon sources

The first goal of this work was to identify carbon sources that repress FK506 production in *S. tsukubaensis*. For this purpose, *S. tsukubaensis* was grown in defined MGm-2.5 medium (Martínez-Castro et al. 2013), a production medium containing starch as main carbon source, glutamate as carbon and nitrogen source and limited in phosphate. This medium supports a good and dispersed growth and high yields of FK506 production. FK506 biosynthesis is triggered after phosphate depletion, which occurs between 80 h-89 h. We selected a set of 8 carbon sources (glucose, fructose, xylose, glycerol, mannitol, maltose, lactose and sucrose) for the study, including the most common repressing sources glucose and glycerol (for a review on *Streptomyces* carbon repression see Ruiz et al. 2010). The presence of the carbon sources in the growth media from the beginning of the fermentation was rejected since growth rate variations might difficult the interpretation of the results (Lounes et al. 1996). The accurate study of the response to carbon source additions requires all cultures to be at the same physiological state

before the addition. Thus, the carbon sources were added during the first growth phase and before the depletion of phosphate (i.e. 70 h).

The repressing effect of a carbon source depends on its concentration; for example, glucose at final concentrations between 1-1.75 % has a positive effect on FK506 production in several *S. tsukubaensis* ZJU01 strains (Chen et al. 2012). Thus, a high final concentration (2.8 % w/v) for all the carbon sources tested was selected for this exploratory experiment. Culture samples for dry weight (from 64 h to 161 h) and FK506 determination (from 92 h to 161 h) were taken. The presence of FK506 in the culture supernatants was tested by agar diffusion bioassays against *S. cerevisiae* TB23. The addition of these carbon sources did not affect growth (data not shown) and only glucose and glycerol inhibited FK506 production (see Table S2). Thus, glucose and glycerol were selected to perform the transcriptomic analysis.

Time-series cultures for transcriptomic analyses

For the transcriptomic analysis, *S. tsukubaensis* was cultured under the same conditions indicated above, adding glucose or glycerol as repressing carbon sources at 70 h. A control condition was included consisting on the addition of maltose, since this disaccharide does not repress FK506 production and is a natural product of starch metabolism. For each experimental condition five replicates were cultured. The final concentrations of glucose and glycerol were established at the same molarity (0.22 M; 2 % w/v and 4 % w/v for glucose and glycerol, respectively). The final concentration of maltose was established at 0.11 M (3 % w/v) in order to equalize the number of glucose molecules available after maltose incorporation. Samples for dry weight, phosphate concentration and FK506 determination were taken between 65 h and 235 h from the five replicates of each culture condition. Samples for RNA extraction were taken at 70 h (immediately before additions), and then from 70.7 h to 148 h (see Materials and Methods).

According to their growth curves and the pattern of phosphate depletion, two cultures from each experimental condition were selected for RNA extraction in order to ensure the highest physiological homogeneity. The growth, phosphate depletion and FK506 production patterns of the six cultures (two from each experimental condition) are depicted in Fig 1. In the control condition, FK506 production started after phosphate depletion (89 h), as expected, since this is the limiting nutrient in this medium. FK506 reached its maximum specific production values at 148 h (see Fig 1b). Glucose addition blocked FK506 production along the whole time curse of the cultures. The addition of glycerol repressed production at least during the first 161 h of culture, although FK506 was detected at the last sample time (235 h).

Immediate response to the repressing carbon sources

In order to identify genes that respond quickly to the carbon source additions, a comparison of the M_g transcription values at 70 h ($t_{70\ h}$) (i.e., immediately before the addition) with the M_g $t_{70.7\ h}$ values (i. e., 40 minutes after the addition) was performed using the limma package. This approach yielded a total of 1176 genes as differentially transcribed after the additions (203 of them with 2-fold or greater changes). In

addition, a regression approach for the first five time-point values ($t_{70.7\ h}$ - $t_{80\ h}$) of each experimental condition was applied using maSigPro to identify genes affected by the additions that might not be detected with the first approach. From this analysis, a total of 1315 genes showed statistically significant differences (255 of them with $R^2 \geq 0.9$). Finally, we focused our functional analysis on a set of 361 genes showing the strongest differences in any of both approaches (203 and 255 for limma and maSigPro, respectively; see Fig S2). These genes are listed in Table S3. The fact that only 63 genes out of 361 showed significant transcriptional variations 40 min after maltose addition supported the choice of this disaccharide as the control addition.

Effects on carbon source transport

First, we focused our attention on the genes encoding the putative transporters for maltose, glucose and glycerol even if most of them were not filtered in the statistical analysis. The maltose ABC transporter genes *malEFG* were downregulated after the three additions, especially after maltose addition (Fig S3.1) and, although not included with the approaches used, the changes were statistically significant $t_{70.7h}$ and t_{72h} for glycerol and maltose conditions, respectively. Transcription of the operon for glycerol transport and metabolism increased after glycerol addition and was transiently downregulated by glucose (Fig S3.2), which was in accordance with the results reported in *S. coelicolor* (Smith and Chater 1988). On the contrary, transcription of the unique glucose permease coding gene, *glcP*, was low throughout the culture and not induced after glucose addition (Fig S3.3), which contrasts with its behavior in *S. coelicolor* (van Wezel et al. 2005). In *S. tsukubaensis*, as well as in *Streptomyces clavuligerus* and *Streptomyces avermitilis*, only one *glcP* gene is found. This gene is orthologue to *S. coelicolor glcP2*, which inactivation does not affect glucose transport in this species (van Wezel et al. 2005). In the upstream region of *glcP* we did not detect bacterial sigma 70 promoters using the online tool BPROM (Solovyev and Salamov 2011). This situation resembles that of *S. clavuligerus*, where the weakly expression of *glcP* accounts for the lack of growth on glucose as the sole carbon source (Pérez-Redondo et al. 2010). Thus, it is possible that transcription of *S. tsukubaensis glcP* depends on a non-constitutive sigma factor and that under our culture conditions an alternative transporter is responsible for the incorporation of glucose. Indeed, two different glucose transporters have been biochemically reported in *Streptomyces lividans* (Hurtubise et al. 1995).

We detected a high number of genes encoding transport-related functions that were affected by the additions (amino acid and oligopeptide transporters related with differentiation are discussed in the corresponding sections). As it might be expected from the concept of CCR, both additions reduced the mRNA levels of genes encoding transporters for alternative carbon sources. This response was significant at $t_{70.7h}$ and t_{72h} for glucose and glycerol additions, respectively (Fig S3.4). Among the affected genes we detected STSU_23336 (homolog to *nagE2*, encoding the predicted N-acetylglucosamine specific IIC component of the PTS system), *dasA* (encoding the chitobiose transporter; Saito et al. 2007) and *msiK* (encoding an ATP-binding protein which is involved in the transport of several carbon sources; van Wezel et al. 1997). Transcription of the xylose transport operon *xylFGH* was also downregulated after the additions (Fig S3.4), which is in contrast to that reported in the model strain (Romero-Rodríguez et al. 2016a). This evidences important metabolic differences between *Streptomyces* strains.

Interestingly, glucose addition exerted a positive effect on several genes related to xylose metabolism. Transcription of two xylose isomerase coding genes (*xylA* and STSU_23777) and other xylose isomerase domain containing genes (i.e. STSU_04768, which was also transiently upregulated after maltose addition) were upregulated by glucose (Fig S3.5). In *S. coelicolor* xylose transport and xylose metabolism genes are regulated independently (Swiatek et al. 2013) and glucose stimulates 10-fold the transcription of the xylose transporter genes *xylFGH* (SCO6009-SCO6011; Romero-Rodríguez et al. 2016a). In *S. tsukubaensis* we observed the opposite behaviour for xylose transporter genes, which were downregulated after glucose addition (see Fig S3.4). This phenomenon might be due to an anticipative regulatory mechanism in which the presence of a common metabolite from glucose and xylose utilization pathways stimulates transcription of genes involved in xylose catabolism. Anticipation to environmental changes is a feature observed in both eukaryots and prokaryots and favoured through the evolution (Mitchell et al. 2009).

Closely located to STSU_04768, we found an ABC transporter operon (STSU_04793-STSU_04803) which was transcriptionally activated after glucose and maltose additions. This operon might encode a ribose transporter and is likely to be regulated by the ROK family transcriptional regulator STSU_04808, which is encoded upstream and showed a similar transcriptional pattern after glucose and maltose additions (Fig S3.6).

Interestingly, transcription of the xylose isomerase coding gene STSU_23777 showed the same transcriptional profile than STSU_23771 (encoding a LysR type regulator) and STSU_23786 (encoding a MarR family regulator; Fig S3.7). The transcription of STSU_23786 showed one of the highest increases detected in mRNA levels after the glucose addition (i.e. 4.7 $\log_2$-fold change).

Effects on central carbon pathways

Glucose addition upregulated the transcription of several genes involved in the glycolytic pathway such as *pfkA3* (coding the 6-phosphofructokinase 3), *tpiA* (coding a triose-phosphate isomerase; this gene was not filtered and, thus, is not included in Table S3) and *pgk* (coding a phosphoglycerate kinase). It also increased the transcription of the gluconate kinase coding gene *idnK* (see Fig 2 and Fig S3.8). This is in agreement to that reported for the orthologue SCO1679 in *S. coelicolor,* although we did not detect upregulation of genes encoding gluconate dehydrogenases (Romero-Rodríguez et al. 2016a). In agreement with these results, we observed a decrease in the mRNA levels of genes involved in the gluconeogenic pathway (i.e. the rate controlling phosphoenolpyruvate carboxykinase encoded by *pck*, the glyceradehyde-3-phosphate dehydrogenase 2 coding gene *gap2* and the fructose-1,6-biphosphate aldolase encoded by *glpX;* see Fig S3.9). Glucose upregulated transcription of genes involved in the formation of pyruvate (pyruvate kinase 2 *pyk2;* this gene was not filtered and thus, is not included in Table S3) and oxaloacetate (phosphoenolpyruvate carboxilase *ppc;* Fig S3.9) but downregulated some genes involved in the tricarboxylic acid (TCA) cycle (i.e. malate oxidoreductase *malS4*, succinate dehydrogenases *sdhB* and *sdhA* and citocrome b subunit *sdhC2;* Fig S3.10). These results are in contrast with those reported for *S. coelicolor* by Romero-Rodríguez and coworkers (2016a), who suggested that TCA enzymes might be regulated by metabolites rather than at the transcriptional level.

The effect of glycerol addition on carbon central pathways was narrower compared to that of glucose. The responses detected were limited to the downregulation of *pgk* (encoding the bifunctional phosphoglycerate kinase from glycolisis), and *bglA2* (encoding a sugar hydrolase similar to the 6-phospho-beta-glucosidase that generates glucose and glucose-6-phosphate; see Fig S3.8 and S3.9. Glycerol upregulated transcription of the TCA gene *fumC* (encoding a fumarase; Fig S3.10). In *Escherichia coli*, FumC is produced only under low iron availability or when superoxide radicals accumulate, whilst in *Bacillus subtilis* the expression is induced by fumarate and repressed by glucose addition (Park and Gunsalus 1995; Ohné 1975).

Both additions exerted a negative effect on the transcription of genes involved in fatty acid degradation and upregulated transcription of genes involved in the biosynthesis of phospholipids or encoding lipases. Glucose and glycerol additions stimulated transcription of genes involved in fatty acid biosynthesis such as *accB, accE* and *fabH* (Fig S3.11). *accB* and *accE* encode an acyl-CoA carboxilase which catalyzes the formation of malonyl-CoA from acetyl-CoA. This enzyme has been reported to be directly involved in the production of pigmented antibiotics in the model *S. coelicolor*, since mutants in *accB* do not produce actinorhodin or undecylprodigiosine (Rodríguez et al. 2001). FabH is a β-oxoacil-CoA synthase III, responsible for the initiation of fatty acid biosynthesis in *S. coelicolor* and *Streptomyces glaucescens* (Revill et al. 2001; Han et al. 1998). *fabH* is part of the operon for fatty acid biosynthesis *fabD-fabH-acpP-fabF*, that shares a very similar transcriptional profile with *accBE*, indicating a common regulation for both operons (Fig S3.11). Transcription of *fabG3*, which is involved in fatty acid biosynthesis in *S. coelicolor* (SCO1346), was downregulated after glucose and glycerol additions. *S. tsukubaensis* contains three *fabG* paralog genes (as well as the model species) that showed very different profiles (Fig S3.12), indicating different transcriptional regulations.

Effects on nitrogen assimilation

Glucose addition stimulated immediately ($t_{70.7h}$) the transcription of *gltB* and *gltD*, which encode the subunits of the L-glutamate synthase, while glycerol produced a steady increase (Fig S3.13). This result is consistent with those observed in *S. coelicolor* and *B. subtilis*, where transcriptions of *gltBD* and *gltAB* are induced by glucose (Gubbens et al. 2012; Blencke et al. 2003). The *gdhD* gene encodes a NAD-glutamate dehydrogenase that is glucose-repressed and it is likely involved in glutamate utilization as energy source (Gubbens et al. 2012). In accordance, the transcription of *gdhD* was downregulated immediately after glucose addition (Fig S3.13). These results suggest that glucose represses glutamate consumption and stimulates its biosynthesis. In agreement with these results, glucose and glycerol downregulated transcription of the glutamate ABC transporter coding genes *gluABCD* (Fig S3.13). Interestingly, this result is in contrast with that observed in *S. coelicolor*, in which glucose stimulates transcription of the glutamate transporter (Romero-Rodríguez et al. 2016a).

Glutamate synthase participates in the NH_4^+ assimilation pathway along with glutamine synthetases. In *S. coelicolor, glnA* and *glnII* encode functional glutamine synthetases (Rexer et al. 2006). In *S. tsukubaensis* the orthologue genes showed a transcriptional upregulation after glucose addition (Fig S3.14), although they were not filtered with the approaches used. The same transcriptional pattern was

detected for *amtB*, encoding an ammonium transporter (Fig S3.14). Thus, glucose might stimulate the incorporation of NH_4^+ from the culture broth, which, in turn, might be previously secreted as a byproduct of glutamate consumption.

Transcription of *glnR*, encoding the main nitrogen transcriptional regulator in *Streptomyces* (Fink et al. 2002), was permanently upregulated after glucose addition, whilst glycerol produced only a transient activation. On the contrary, transcription of *glnRII*, a second nitrogen transcriptional regulator, was mainly stimulated after glycerol addition (Fig S3.14).

Effects on sulfate and phosphate assimilation

The three carbon sources, but mainly glycerol, activated the sulfate reduction assimilatory pathway. The *cysHCDN* operon, involved in the transformation of sulfate to sulfite, increased its mRNA levels at $t_{70.7\ h}$ (glycerol addition) or $t_{72\ h}$ (glucose addition). A similar pattern was shown for the adjacent genes *sirA*, which product catalyses the reduction of sulfite to sulfide (Fischer et al. 2012), and the STSU_06028-STSU_06043 operon, which encodes a Nit/Tau family transport system (Fig S3.15). Nit/Tau family transporters are related to the incorporation of nitrates, bicarbonate, taurine or aliphatic sulfonates. Genes for a second Nit/Tau family transporter (STSU_03564-03574), a hypothetical protein (STSU_03554), a sulfatase (STSU_03559) and a Crp family transcriptional regulator (STSU_03579) showed the same profiles (Fig S3.16). Both transporters (STSU_06028-STSU_06043 and STSU_03564-03574) show homology to the *tauABCD* system of *E. coli*, which is involved in the incorporation of sulfonates under sulfur starvation (van der Ploeg et al. 2001). The transcriptional profiles of the second transporter (STSU_03564-03574) indicate a carbon source dependent induction, whilst genes encoding the first transporter already showed high transcription values before carbon source addition. The effect of carbon sources on the transcription of these genes might reflect a stimulation of sulfur assimilation by a rich nutritional status.

In a similar manner, the carbon sources stimulated phosphate transport, since all the additions increased transcription of the phosphate transporter encoded by the *pstSCAB* operon (Fig S3.17). The *phoRP* operon (encoding the two-component system that governs the *pho* regulon; Wanner 1993) and the divergent *phoU*, showed a similar transcriptional pattern, although their transcriptional activation was significant only after glucose and glycerol additions (Fig S3.17). Finally, transcription of STSU_16912, which is likely to encode a phosphatase and belongs to the *S. coelicolor pho* regulon (SCO3790; Sola-Landa et al. 2008) showed an equivalent transcriptional pattern, although the increase was significant only after glucose addition (Fig S3.17). The transcriptional induction of phosphate transporters and scavengers suggests an increased need of phosphate for the transport and metabolism of the carbon sources. Moreover, this is a new evidence of the cross-regulation between carbon and phosphate metabolism, which has been documented before. For example, in *S. lividans* PstS is accumulated in the media in the presence of certain carbon sources (Díaz et al. 2005); in *S. coelicolor* transcriptions of *glpQ1* and *glpQ2* (encoding glycerophosphodiester phosphodiesterases) are regulated not only by phosphate concentration but also by the carbon sources present in the medium (Santos-Beneit et al. 2009).

Effects on amino acid metabolism

Carbon source additions affected the transcription of amino acid metabolism genes differently, some of these changes were limited to a transient activation and some were more drastic and permanent. Glucose activated the transcription of genes involved in aspartate catabolism (i.e. the *ask-asd* operon, Fig S3.18), histidine synthesis (i.e. *hisCBHAF*; *hisD* and *hisI* genes lack valid probes in the microarrays; Fig S3.19). Although not filtered with our approaches, we observed that tryptophan biosynthetic genes were slightly upregulated by glucose and glycerol (i.e. *trpE*, *trpC* and *trpBA;* Fig S3.20).

The biosynthetic pathway of serine, glycine, threonine and methionine is depicted in Fig 3. Glucose increased at $t_{70.7h}$ the mRNA levels of the D-3-phosphoglycerate dehydrogenase coding gene *serA* (serine biosynthesis), the L-threonine 3-dehydrogenase coding gene *tdh*, and the 2-amino-3-ketobutyrate coenzyme A ligase coding gene *kbl* (involved in threonine-glycine interconversion; see Fig S3.21). The first step of serine biosynthesis, catalized by the *serA*, is also the gate to the biosynthesis of threonine and glycine (see Fig 3). In addition, serine is the precursor of the sulfur-containing amino acids cysteine and methionine (Fig 3).

All the carbon sources upregulated the transcription of cysteine and methionine metabolic genes. The transcription values of *cysM* (encoding a cysteine synthase that converts acetyl-L-serine and sulfide into cysteine) indicate a constitutive high transcription that is activated at $t_{70.7h}$ and t_{72h} after glycerol and glucose additions, respectively (Fig S3.22). It is worthy to mention that in the *S. tsukubaensis* genome, we found two *cysM* orthologues, STSU_31680 and STSU_15012, and the last one showed an *fkb*-like transcriptional profile (Fig S3.22).

Glucose and glycerol additions increased the transcription of two putative cysteine dioxygenase coding genes (STSU_22610 and STSU_08058; Fig S3.23). Although distantly located, the close similarity of their profiles through all the time series (not shown), indicated a coordinated regulation. Cysteine dioxigenases convert cysteine to L-cysteine sulfinate, which, in mammals is used for the generation of piruvate and sulfate (by aspartate aminotransferase activity) or hypotaurine (by cysteine sulfinic acid decarboxylase activity). In bacteria no cysteine sulfinic acid decarboxylase activity has been reported and, thus, it seems unlikely that cysteine sulfinate acts as precursor for taurine formation (Dominy et al. 2006).

The *S. tsukubaensis* genome contains two aspartate aminotransferase coding genes (*aspC* and STSU_27731). Both of them showed a transcriptional activation at $t_{70.7h}$ after glycerol addition although they were not filtered (Fig S3.24). Thus, glycerol addition might enhance the flux from cysteine to piruvate and sulfate. Glycerol addition stimulated the formation of L-methionine from L-homoserine and Acetyl-CoA (see Fig 3) through the transcriptional upregulation of *metH* (encoding a 5-methyltetrahydrofolate-homocysteine S-methyltransferase) and STSU_01830-STSU_01835, which is likely to encode the *metBX* operon (Fig S3.25).

Glucose and glycerol additions increase transcription of stress response genes

Both glycerol and glucose additions stimulated transcription of several genes involved in oxidative stress response at $t_{70.7\,h}$ and $t_{72\,h,}$ respectively (*ahpC*, *ahpD* and *oxyR*; Fig S3.26a). Genes *ahpC*, *ahpD* encode alkyl hidroperoxyde reductases and are directly activated by the transcriptional regulator OxyR (Hahn et

al. 2002). These three genes maintained significantly higher mRNA levels during the FK506 producing phase after glucose and glycerol additions than in the control condition. In fact, mRNA levels decreased after 89 h in the maltose added cultures (Fig S3.26b). Glycerol addition increased specifically the transcription of several genes involved in disulphide stress response at $t_{70.7\ h}$ such as the regulatory operon *sigR-rsrA* and the thiorredoxin and thiorredoxin reductase coding genes *trxA* and *trxB*. The thioredoxin coding gene *trxC* was also upregulated at $t_{70.7\ h}$ after glucose addition (Fig S3.26). Nevertheless, these changes were transient and the mRNA levels of these genes during the FK506 production phase were similar in the three experimental conditions (data not shown).

The main source of oxidative stress in the cultures might be the activity of the respiratory chain. For example, in *E. coli* as much as 87 % of the H_2O_2 is generated by this mean (Gónzalez-Flecha and Demple 1995). Thus, the activation of genes involved in oxidative and disulphide stress might reflect an increased flux through the respiratory chain. Any of the three additions increased the mRNA levels of the NAD^+ synthase coding gene *nadE*, which might indicate a situation of low NAD^+ availability. We also observed a downregulation in the transcription of the *nuo* operon, encoding the NADH dehydrogenase I, which is responsible for the regeneration of NAD^+ in the respiratory chain. The repression was stronger in the case of glycerol addition (Fig S3.27). Considering that in *E. coli*, the *nuo* operon is repressed in anaerobic conditions, but also under high glycolytic fluxes (Vemuri et al. 2006), these results are in agreement with the increased respiratory activity suggested above.

Nucleotide metabolism, transcription and translation

Glucose activated rapidly transcription of genes for *de novo* biosynthesis of pyrimidines from L-glutamine to UMP (*pyrR*, *pyrBC*-STSU_29616-*pyrAa*-*pyrA*-*pyrD*, *pyrF*; Fig S3.28). Genes for the biosynthesis of purines (operon *purNH*) were transiently repressed by glucose and glycerol at $t_{70.7\ h}$, and activated at $t_{72\ h}$ by glucose (Fig S3.29). Meanwhile *deoD* (STSU_12420), which product is involved in the nucleotide salvage pathway, showed just the opposite profile (Fig S3.29). Besides, transcription of adenylate kinase gene *adk* was permanently upregulated after glucose addition (Fig S3.30). The encoded enzyme contributes to the homeostasis of adenine nucleotides catalyzing the reversible reaction ADP+ADP $\leftrightarrow$ ATP+AMP.

The effect of glucose was extended to genes related with transcription and translation processes. It stimulated transcription of *rpoA* and *rpoC* at t_{72h}, encoding subunits of the RNA polymerase (Fig S3.31; *rpoA* was not filtered in the analysis but showed the same transcriptional profile than *rpoC*) and up to 35 genes encoding ribosomal proteins (Fig S3.32 and Fig S3.33; note that not all these genes were filtered in the analysis but they share the same profile). In addition, we detected several genes activated by glucose that did not pass the filters used in the analysis such as the translation initiation factor gene *infA* (Fig S3.34), the pseudouridine tRNA synthase *truA* and the phenylalanine tRNA ligase *pheST* operon (Fig S3.34). All the additions activated the transcription of *prfB*, encoding the peptide chain release factor 2 (Fig S3.34).

Glucose promoted mRNA turnover to adapt the transcriptome to a new metabolic background. For example, the mRNA levels of *rns* (which encodes the ribonuclease E, a protein likely to be part of a RNA degradosome-like complex in *S. coelicolor*; Lee et al. 2003) increased after the addition (Fig S3.35). It also stimulated transcription of STSU_18582, encoding an ATP-dependent RNA helicase (Fig S3.35).

Several heat shock proteins (Hsps) that serve as molecular chaperones or proteases were induced after the additions. Hsps are not only involved in the stress response, they also play crucial roles under normal conditions by assisting in the folding of new polypeptides (Hartl 1996). Thus, the additions might stimulate the formation of new polypeptides. Transcription of the chaperone coding gene *groEL*, which is induced under acidic and heat stress (Kim et al. 2008; de León et al. 1997), was significantly downregulated after all the additions (at $t_{70.7\,h}$ for glucose addition and at $t_{72\,h}$ for glycerol and maltose additions). The mRNA levels of *hspR*, encoding the heat-shock regulatory system regulator, increased after all the additions and, as expected, the transcriptional profile of the target operon *dnaK-grpE-dnaJ*- was very similar (Fig S3.36; Bucca et al. 2009). In addition, the protease coding gene *lon*, which is a direct target of HspR (Bucca et al. 2003) increased its transcription after all the additions (Fig S3.36).

Interestingly, two hours after glucose addition transcription of *pcrA* was specifically downregulated. This gene encodes the proteasome subunit alpha. We also found a set of genes related to the proteasome complex (*pcrB*-STSU_28817-STSU_28822-*arcAA*) that were specifically downregulated by glucose, although they were not filtered with our approach (Fig S3.37). It must be noted that a link between proteasome and stress-responsive proteins has been suggested before since mutant strains show an increased resistance to certain hydroperoxides (De Mot et al. 2007). Thus, transcriptional downregulation of the proteasome coding genes might give an advantage under the oxidative stress situation generated by the additions.

Transcriptomics during the stationary growth phase: effects of the carbon sources on antibiotic production and morphological differentiation

Transcriptional patterns of the fkb *cluster genes*

The transcriptomic profiles of the *fkb* cluster under the control condition allowed us to identify different transcriptional patterns (see Fig 4) which correlate well with the transcriptional units proposed by Ordoñez-Robles and coworkers (2016). Transcription of *fkbR* (encoding a LysR transcriptional regulator) and the *all* subcluster genes *allMNPOS* was low throughout the temporal series, in accordance with that reported before (Ordóñez-Robles et al. 2016). The operon *tcs6-fkbQ-fkbN*, which is transcribed in a single mRNA from two independent promoters (one *fkbN*-dependent and other *fkbN*-independent; Ordóñez-Robles et al. 2016), increased its transcription preceding FK506 production in a two-phase fashion: first from 80 h to 89 h (corresponding to phosphate depletion), and later from 92 h to 100 h. The rest of genes, encoding most of the structural genes, showed a transcriptional activation following the increase in *fkbN* mRNA levels (i.e. from 92 h), which is in agreement with their FkbN dependency (Ordóñez-Robles et al. 2016). In view of these results we can conclude that glucose and glycerol exert their effect on FK506 production at least at the transcriptional level. Considering that *fkbN* transcription is not strongly

autoregulated (Ordóñez-Robles et al. 2016), a key transcriptional regulator or sigma factor might be absent under our repressing experimental conditions.

Effects on genes related to morphological and biochemical differentiation

The addition of the carbon sources downregulated permanently the transcription of genes involved in biochemical and physiological differentiation. Transcription of the RNA polymerase sigma factor coding genes *hrdA* and *bldN* was downregulated at $t_{70.7h}$ after glucose and glycerol additions (Fig S3.38a) and the decrease in their transcription levels was maintained throughout the cultures (Fig S3.38b). Transcription of *hrdA* correlates with the formation of aerial mycelia in *Streptomyces aureofaciens* (Kormanec and Farkasovský 1993) and might control secondary metabolism genes (Strakova et al. 2014). BldN is part of the signaling cascade that leads to morphological differentiation in the genus *Streptomyces* and its repression by glucose has been reported previously (Gubbens et al. 2012; Romero-Rodríguez et al. 2016a).

The BldK transporter is considered to be involved in the detection of the signal leading to morphological differentiation in this genus (Nodwell et al. 1996). Transcription of the *bldK* operon was downregulated at t_{72h} after glucose and glycerol additions (Fig S3.39a) and this response was maintained through the culture (Fig S3.39b). A second oligopeptide transporter operon (STSU_09304-STSU_09324) was negatively regulated at $t_{70.7h}$ after glucose addition (Fig S3.40a and b). This transporter has been shown to be related with morphological differentiation in *S. coelicolor* and repressed by glucose (Park et al. 2005; Romero-Rodríguez et al. 2016b).

Glucose addition affected specifically transcription of *wblA*, encoding a key factor for sporulation in several *Streptomyces* species (Rabyk et al. 2011; Kang et al. 2007; Fowler-Goldsworthy et al. 2011). We also detected an increase in transcription of *obg* after glucose and glycerol additions (Fig S3.41a and b). This gene encodes a membrane-bound GTPase which avoids aerial mycelium formation in *S. coelicolor* (Okamoto and Ochi 1998). Obg proteins act as sensor of the energetic status of the cell and serve as conectors among different pathways (reviewed by Kint et al. 2014).

Crp is a key player of *E. coli* CCR and a master regulator of antibiotic production in *S. coelicolor* although it seems to be not involved in *Streptomyces* CCR (Gao et al. 2012). Thus, given its relevance, we search for *S. tsukubaensis* genes encoding regulators from the Crp family and identified three genes: *crp*, *eshA* and STSU_03579 (Fig S3.42a and b). *crp* showed a constitutive transcription whilst transcription of STSU_03579 was transiently upregulated by glycerol. Glucose and glycerol additions decreased the mRNA levels of *eshA*, which product regulates antibiotic production in *S. coelicolor* and *Streptomyces griseus* (Kawamoto et al. 2001; Saito et al. 2006). The two genes located immediately after *eshA* (STSU_03589 and STSU_03594) showed the same transcriptional pattern (Fig S3.42). In *S. coelicolor*, their ortholog genes are involved in the biosynthesis of the volatile metabolite methyl-isoborneol (Wang and Cane 2008).

fkbN-*like transcriptional profiles*

Genes showing transcriptional profiles similar to that of *fkbN* might be involved in FK506 production or precursor supply and thus, they might be useful candidates for genetic engineering of the strains to strength production of this macrolide. In order to find such candidates, we searched for genes showing a transcriptional profile with a Pearson correlation coefficient equal or higher than 0.9 respect to the transcriptional profile of *fkbN*. By this means we identified 80 genes that are summarized in Table S4.

Among the genes predicted to encode proteins with a regulatory role related to morphological differentiation, we identified *ramR* (Fig S3.43), whose product controls the expression of the *ram* operon, involved in the transition from vegetative to aerial growth in *S. lividans* (Keijser et al. 2002). Transcription of *atrA* showed also an *fkbN*-like profile (see Fig S3.43). AtrA is a TetR transcriptional regulator that activates transcription of the pathway specific regulators *actII-orf4* and *strR* in *S. coelicolor* and *S. griseus*, respectively (Uguru et al. 2005; Vujaklija et al. 1993). It also regulates in a positive manner the daptomycin cluster of *Streptomyces roseosporus* (Mao et al. 2015).

As a second approach, we focused our attention in the transcriptional profiles of the orthologues of well known *S.coelicolor* secondary metabolism regulators (reviewed by van Wezel and McDowall 2011). The transcriptional patterns of those showing a positive correlation with the transcription of the *fkb* cluster (see Table S5) are depicted in Fig S3.44 and S3.45. Among them, *afsR* is an interesting candidate for further studies since it has been found to be overexpressed in a *S. tsukubaensis* FK506-overproducing strain (Du et al. 2014).

Among the genes encoding biosynthetic functions related to the secondary metabolism and showing *fkbN*-like profiles we identified STSU_07618 and *ppt1* (Fig S3.46). These genes encode a type II thioesterase and a 4'-phosphopantetheynil transferase which transcription has been reported to be affected by FkbN inactivation (Ordóñez-Robles et al. 2016). In addition, the product of the *ppt1* orthologue is involved in FK506 production in *S. tsukubaensis* L19 (Wang et al. 2016). We also identified a *whiE* gene which is related to the production of the spore pigment (Davis and Chater 1990).

DISCUSSION

In this work we report for the first time that glucose and glycerol block FK506 production in *S. tsukubaensis*. The lack of transcriptional activation of the *fkb* cluster indicates that both sugars exert their role at least at the transcriptional level. To our knowledge, this is the first report on the repressing role of glucose in *S. tsukubaensis*, since Yoon and Choi (1997) reported no differences in FK506 production in liquid cultures containing glucose 0.17 M (3 % w/v) and Martínez-Castro and coworkers (2013) did not detect carbon repression of FK506 biosynthesis on ISP4 liquid media in the presence of glucose 0.22 M (2 % w/v). Nevertheless, the differences in media composition and the presence of glucose from the beginning of the cultures might account for such different results.

This work represents the first genome-wide study on the effects of glycerol as a repressing carbon source in *Streptomyces*. Using a second repressing carbon source enables us to distinguish between general and specific regulatory mechanisms. In fact, we identified common transcriptional patterns but also different responses between glucose and glycerol experimental conditions and we can conclude that the effect of

glycerol on central carbon pathways is much narrower than that of glucose. Both sources stimulated transcription of genes involved in DNA replication and transcription and, as expected from the concept of CCR, downregulated genes encoding alternative carbon source transporters. Several genes related to sulfate and phosphate assimilation increased their mRNA levels in response to the additions, highlighting the importance of cross-regulation between nutritional networks. Glucose and glycerol decreased transcription of key genes involved morphological and biochemical differentiation throughout the cultures. As it has been suggested before for glucose (Romero-Rodríguez et al. 2016b), preferred carbon sources might block the signaling cascade leading to differentiation at very early stages such as the transport of certain oligopeptides. As in the model species, we identified a permanent transcriptional repression of the genes encoding the oligopeptide transporters *bldK* and STSU_09304-STSU_09324 (othologue to SCO5480-SCO5476). Interestingly, although both operons share a similar transcriptional pattern along the cultures, the response to carbon addition of STSU_09304-STSU_09324 was fastest than that of *bldK*. The predicted products of the lipoprotein coding genes of both transporters show a 27.8 % of identity and a 43.8 % of similarity. Thus, we consider this transporter as a new promising candidate for the study of differentiation in *Streptomyces*. Nevertheless, the lack of transcription of key developmental and *fkb* genes is likely to be related with the absence of certain transcriptional regulators or sigma factors such as *hrdA* or *bldN*.

In the case of glucose several omic studies are available to compare our results. We obtained experimental evidence supporting the different transcriptional regulation of paralog genes encoding the same enzymatic activity such as the *pfkA*, *gdh* and *fabG* genes. It is worthy to mention the differences detected between *S. tsukubaensis* and the model species *S. coelicolor*. For example, glucose increases transcription of the glutamate transporter operon *gltABCD* in *S. coelicolor*, since glutamate is preferred over glucose in this species (Romero-Rodríguez et al. 2016a; van Wezel et al. 2005). In *S. tsukubaensis* we observed the opposite response, indicating that glucose slows down glutamate consumption and might act as preferred carbon source over glutamate. Similarly, the transcriptional behavior of the xylose transporter genes is opposed in both species (Gubbens et al. 2012; Romero-Rodríguez et al. 2016a). In addition, contrary to the situation in the model species, there is a lack of glucose-dependent transcriptional activation of the glucose permease coding gene *glcP* in *S. tsukubaensis*. This raises the question of how is glucose internalized in this species. These examples reflect the differences between regulatory networks in *Streptomyces* species and strengthen the utilization of new models to unravel *Streptomyces* biology.

This work also highlights the importance of performing time series designs instead of one point designs when analyzing omic data. For example, Romero-Rodríguez and coworkers (2016b) did not detect differences in the expression of the important transcriptional regulator AtrA (SCO4118) between repressing and non-repressing conditions in the unique sample collected during the exponential growth phase. In our work, we did not detect differences between experimental conditions during the exponential growth phase, but the mRNA levels of the orthologous *atrA* gene (STSU_07858) were 3.2 times higher in the control than in repressing conditions during the stationary phase (i.e. 100 h). Therefore, relevant information might be lost in one point designs.

Finally, the identification of transcriptional regulators showing *fkbN*-like transcriptional profiles that are involved in antibiotic production in other *Streptomyces* species (i.e. *atrA* or *afsR*) provide candidates for FK506 yield improvement but also for the awakening of secondary metabolite cryptic clusters.

Conflict of interest

The authors declare no financial or commercial conflict of interest

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Figure legends

Fig 1. Growth, FK506 production and phosphate depletion patterns in the cultures. A) Growth is represented as the average of the dry weight values from two replicates (glucose, glycerol and maltose supplemented conditions are represented with rhomboids, circles and squares, respectively). B) FK506 production in each culture broth. C) Phosphate depletion pattern in each culture. Note that phosphate is depleted between 80 h and 89 h, since its concentration fells under 100 μM in all the replicates. For panels B and C the two replicates of glucose (rhomboids), glycerol (circles) and maltose (squares) supplemented cultures are represented with black and grey lines.

Fig 2. Schematic representation of central carbon pathways and the effect of glucose addition on involved genes. Genes showing transcriptional upregulation (↑) or downregulation (↓) are indicated. Note that for the step in which several paralogs are involved, only those significantly affected are depicted.

Fig 3. Schematic representation of the serine, glycine, threonine and methionine biosynthetic pathways and the effect of glucose addition. Genes showing transcriptional upregulation are indicated above the corresponding arrow.

Fig 4. Gene organization of the *fkb* cluster (a) and transcriptional patterns detected under the three experimental conditions (b). In panel A, the transcriptional units detected by Ordóñez-Robles et al. (2016) are indicated in black frames. In panel B, the average M_g values of selected genes are depicted, except for *fkbR* and *fkbG*, which are represented independently. In the representation of average M_g values error bars have been omitted to facilitate the visualization of the results. Maltose, glucose and glycerol conditions are represented in black, red and blue lines, respectively.

Fig S1. Validation of microarray data by RT-qPCR. The correlation between the $\log_2$ fold changes in the transcript levels of target genes obtained by microarray and RT-qPCR is represented. Comparisons were performed between 89 h samples from different culture conditions (i. e. glucose vs maltose) but also between time samples from the same experimental conditions (i. e. 89 h and 70 h from glucose cultures).

Fig S2. Representation of the group of the genes selected for the analysis. The statistical analysis with limma (blue circle) yielded 203 genes significantly affected after the additions with 2-fold or greater changes at 70.7 h. The statistical analysis using maSigPro (red circle) revealed differences in 255 genes showing $R^2 \geq 0.9$. The functional analysis was focused on the set of 361 genes listed in Table S3.

Fig S3. Transcriptional profiles of selected genes. The $\log_2$ transcription values (M_g) of selected genes along the cultures are depicted for the three experimental conditions. From left to right, the panels correspond to the glucose, glycerol and maltose (control) conditions. For some figures, we depict a forth panel on the right indicating the average M_g transcription values of selected genes under the three experimental conditions to facilitate the comparison of the profiles (in this case, glucose, glycerol and maltose additions are represented as red, blue and black lines). Error bars are omitted to facilitate the

visualization of the results. In figures 4.11 and 4.19, transcription of *fabD*, *hisD* and *hisI* is not represented since the microarrays used lacked probes for these genes.

Figure 1

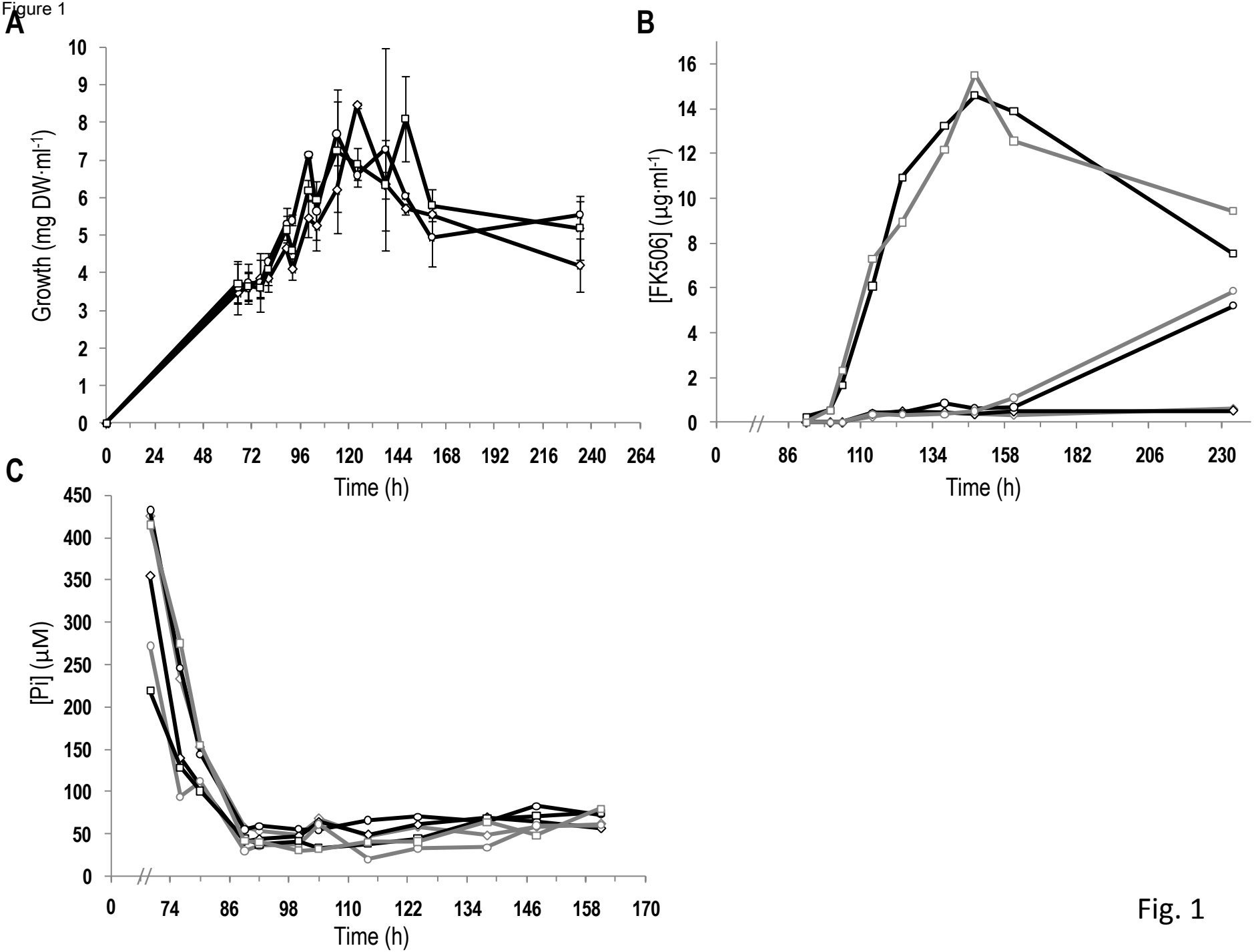

Fig. 1

Figure 2

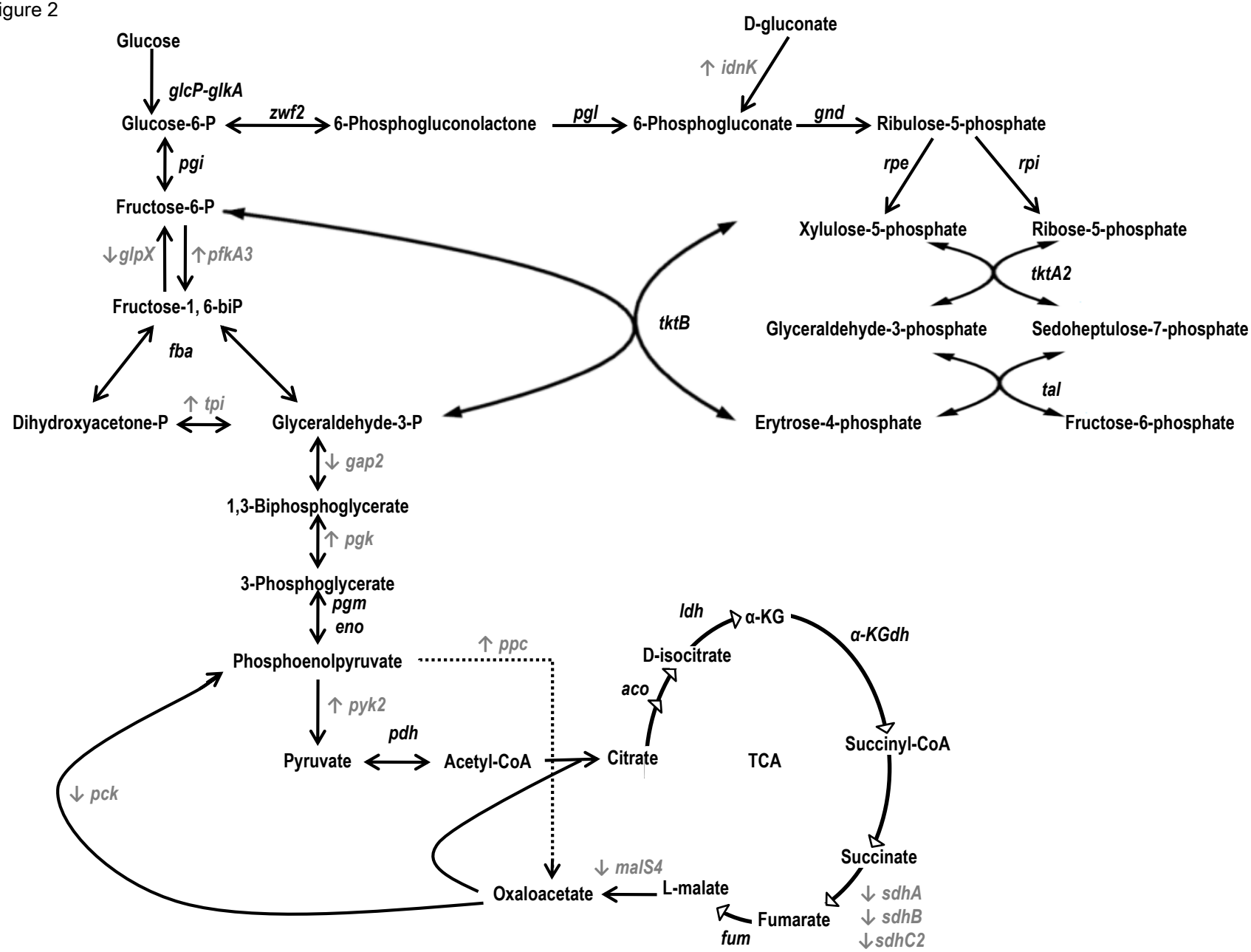

Fig. 2

Figure 3

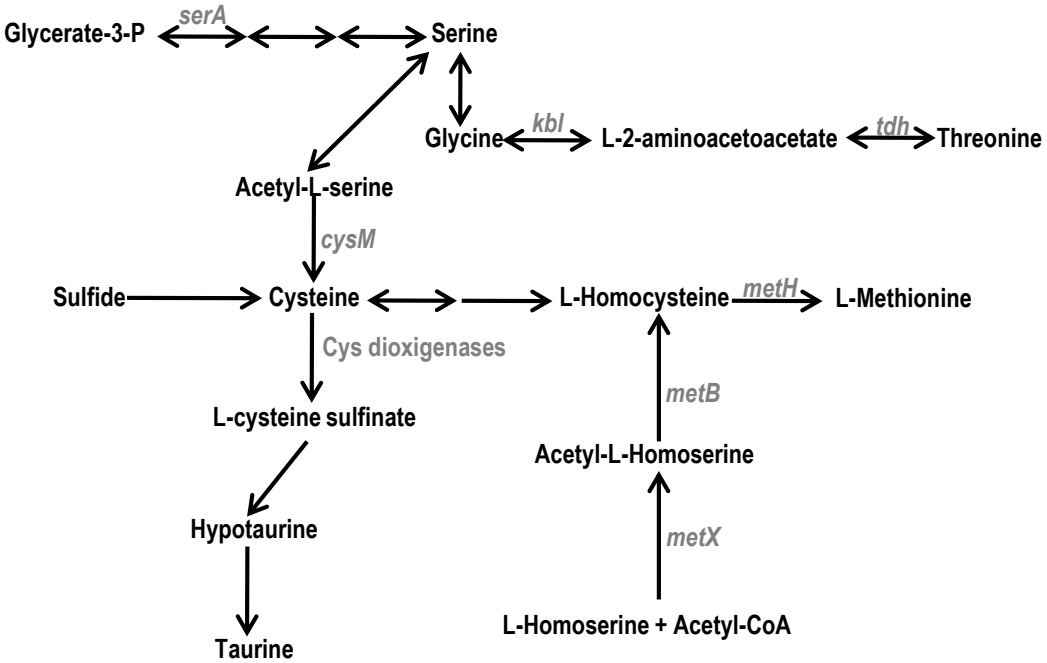

Fig. 3

Figure 4

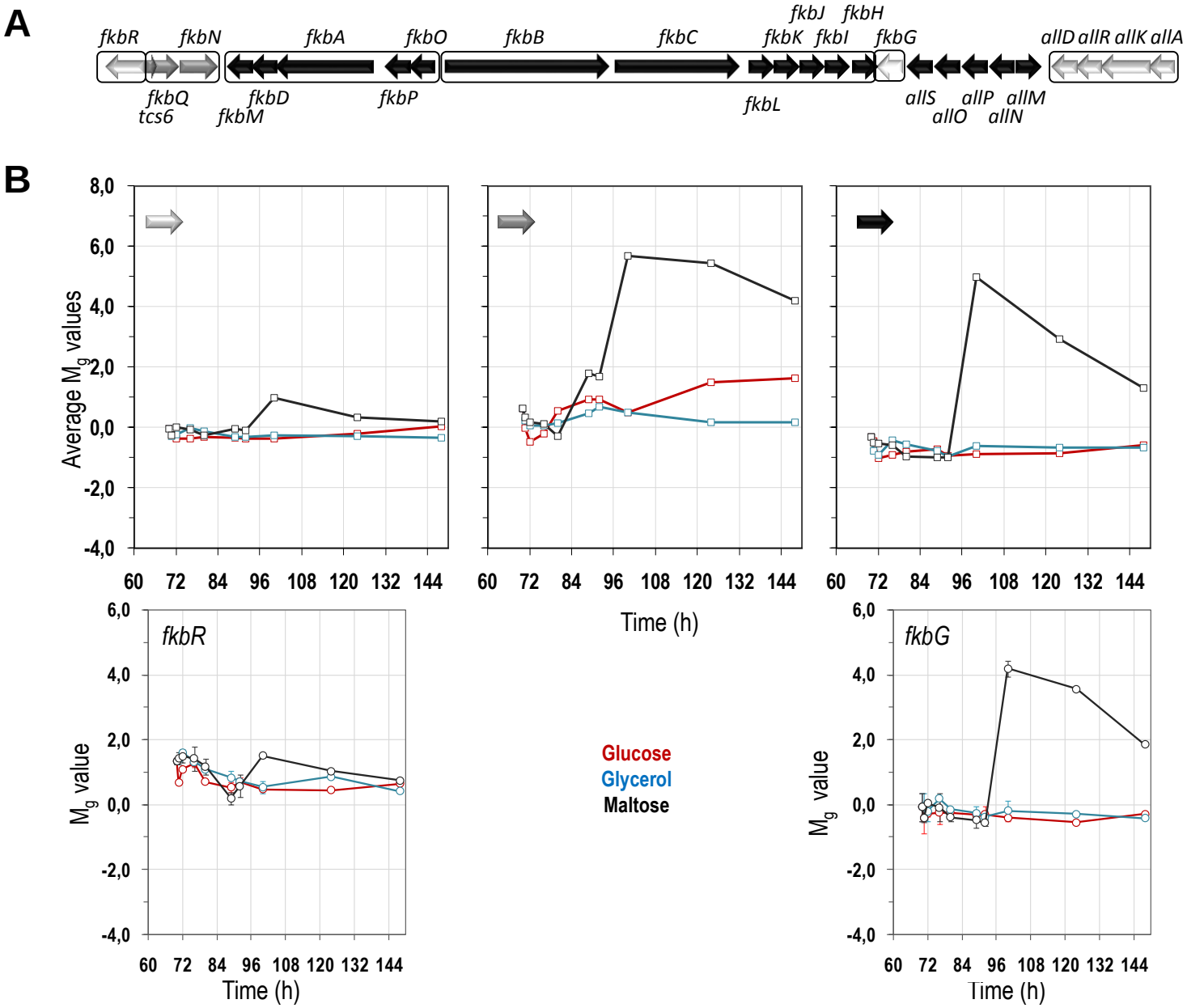

Fig. 4

Table S5

Fig S1

Fig S2

Fig S3