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Mutagenesis of the *tal* gene-encoding Transaldolase in the Cyanobacterium, *Anabaena* sp. PCC7120

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Abstract: The transaldolase gene (*tal*) of *Anabaena* sp. PCC7120 was interrupted by the insertion of the interposon Ω. Transaldolase assays showed that the *tal* mutant strain possessed the same activity as the wild-type, indicating that the second copy of the gene complements the enzyme activity. Being coded by a gene (*zwf*) downstream of *tal* and probably in the same operon, glucose-6-phosphate dehydrogenase (G6PDH) activity was also analysed. Only 34% of wild-type G6PDH activity was retained in the *tal* mutant strain. This may have due to the polar affect of *tal* mutation on the transcription of the *zwf* gene. Growth of the *tal* mutant was not different than that of the wild-type in the presence of combined nitrogen, but the mutant reached the stationary phase faster than the wild-type in the absence of combined nitrogen. This was probably because of the reduction of G6PDH activity, resulting in less production of reductant and energy in heterocysts, which negatively affects nitrogen fixation and growth.

Key Words: Transaldolase mutant, *tal* mutation, glucose-6-phosphate dehydrogenase, *Anabaena*

Siyanobakteri *Anabaena* sp. PCC7120 Suşunun Transaldolazı Kodlayan *tal* Geninin Mutasyonu

Özet: *Anabaena* sp. PCC7120 transaldolaz geni (*tal*) Ω interpozonu eklenerek mutasyona uğratılmıştır. Transaldolaz testleri mutant ve yabani tip hücrelerin aynı düzeyde aktiviteye sahip olduğunu göstermiştir. Bu sonuç bu genin genomdaki diğer kopyasının enzim aktivitesini tamamladığını göstermektedir. *tal* geninin aşağısında ve muhtemelen aynı operonda yer alan bir gen (*zwf*) tarafından kodlanması nedeniyle ayrıca glukoz-6-fosfat dehidrogenaz (G6PDH) aktivitesi de test edilmiştir. Bu *tal* mutant suşta G6PDH aktivitesinin sadece %34'ü korunmuştur. Bu durum *tal* mutasyonunun *zwf* geninin transkripsiyonu üzerine polar etkisinden kaynaklanmış olabilir. Kullanılabilir azot varlığında *tal* mutantların gelişmesi yabani tiplerden farklı değildir. Fakat kullanılabilir azot yokluğunda mutant, yabani tipten daha erken durgunluk fazına ulaşmıştır. Bu durum muhtemelen heterosistlerde düşük G6PDH aktivitesine bağlı olarak daha az indirgeyici güç ve enerji üretilmesi ve sonuçta azot fiksasyonu ve gelişmenin olumsuz etkilenmesinden kaynaklanıyor olabilir.

Anahtar Sözcükler: Transaldolaz mutantı, *tal* mutasyonu, glukoz-6-fosfat dehidrogenaz, *Anabaena*

Introduction

Cyanobacteria produce their maintenance energy during dark periods by the dissimilation of the glucose produced during light periods via photosynthesis, which is stored as glycogen. This dissimilation is carried out through the reactions of the oxidative pentose phosphate (OPP) cycle (1). The enzymes of the OPP cycle are highly active in heterocysts (2,3), and supply reducing power and energy for nitrogen fixation (4,5). The cycle reduces $NADP^+$ in the first 2 steps, which are catalysed by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase. These NADPH molecules are used either to produce ATP by oxidative phosphorylation (6) or to donate electrons to the nitrogenase system (5). The other reactions of the cycle act to convert 5-carbon sugars to 6-carbon sugars, which enter the cycle again. This action provides the complete breakdown of glucose and production of the maximum amount of NADPH (1). Thus, the OPP cycle has an important role in the metabolism of cyanobacteria vegetative cells and heterocysts.

Transaldolase (EC 2.2.1.2) is a component of the OPP cycle, catalysing the transfer of a C_3 unit from sedoheptulose-7-phosphate to glyceraldehyde-3-

phosphate, yielding fructose-6-phosphate and erythrose-4-phosphate (7). The complete breakdown of glucose is vital to cyanobacterial cells under nitrogen-limited conditions, as well as in the dark. Two NADPH molecules are produced for each cycle, releasing 1 molecule of CO_2. Thus, the complete breakdown of 1 molecule of glucose via the complete operation of the cycle provides 12 NADPH molecules that can be used as a source of energy and/or reductant to nitrogenase under the limitation of combined nitrogen.

Despite this importance, transaldolase is not well characterised in cyanobacteria. About 4 decades ago Latzko and Gibbs assayed transaldolases from the cyanobacterium, *Tolypothrix tenuis* (*Calothrix* sp. PCC7101), and from spinach (8). Cyanobacterial cells had 4-fold higher activity than spinach cells; 1.55 and 0.4 mmol mg^{-1} of protein h^{-1}, respectively. Later, it was suggested that the enzyme was a component involved in carbon dissimilation through the OPP cycle in cyanobacteria (1).

The existence of *tal* genes in *Anabaena* sp. PCC7120 and *Nostoc* sp. ATCC29133 was suggested based on relatively poor sequence homologies in the early 1990's (9,10). Eventually, results of genome projects showed that 2 copies of the *tal* gene are present in the genome of cyanobacterial strains (11,12).The translation products of these copies, namely *tal1* and *tal2*, are not identical and show poor homology. A *talB* mutant *Escherichia coli* (7) showed no transaldolase activity, while no effect on growth was found in glucose minimal medium, indicating absence of a second copy of the transaldolase gene.

In *Anabaena* sp. PCC7120, the *tal2* copy of the gene is located between the *fbp* and *zwf* genes. The genes in this region (*fbp-tal2-zwf-opcA*) were reported to be members of a single operon and had transcription signals that began from different points of the operon in the *Nostoc punctiforme* strain, ATCC29133 (13). The *Anabaena* sp. PCC7120 genome shows the same gene arrangement in this region; therefore, a mutation in *tal2* might probably affect the downstream genes, *zwf* and *opcA*. Consequently, mutagenesis of the *Anabaena* sp. PCC7120 *tal2* gene was attempted in order to reveal the function of transaldolase in carbon and nitrogen metabolism, and to determine if it affects the downstream *zwf* gene in *Anabaena* sp. PCC7120.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Anabaena sp. PCC7120 was grown in liquid BG11 medium at 30 °C under white fluorescent light (20 µE m^{-2} s^{-1}) (14). *Escherichia coli* strains used in this study were grown in LB medium and 2 × YT medium at 37 °C (15). The bacterial strains and plasmids used in this study are listed in Table 1.

DNA Manipulations

Chromosomal DNA from *Anabaena* sp. PCC7120 was purified on a small scale, using a method described by Cai and Wolk (23). Plasmid isolation from *E. coli*, and restriction digestion and transformation in *E. coli* were performed using standard molecular biological techniques (15).

Plasmid DNA was conjugated into *Anabaena* sp. PCC7120 cells by triparental mating, using a method described by Elhai and Wolk (20). A triparental mating procedure was performed to conjugate plasmid pAUG401 into *Anabaena* sp. PCC7120 wild-type cells. Of the 3 parents, 1 *was E. coli* AEE101 carrying conjugative plasmid RP-4, which provides the genes for conjugative apparatus and functions. The other parent was *E. coli* HB101, carrying helper plasmid pRL528, which contains the *mob* gene for nicking the *oriT* region. Plasmid pAUG4001 cannot replicate in cyanobacterial cells because it lacks a cyanobacterial replicon. The third parent was *Anabaena* sp. PCC7120 wild-type cells. The plasmid was transformed in *E. coli* HB101 and triparental mating was performed mixing these 3 cells on filter paper placed onto BG11 medium.

A homologous DNA probe was labelled with [^{32}P]dCTP, using the nick translation method, according to the manufacturer's instructions (Bohringer, Mannheim). This probe was used to detect a particular DNA in wild-type and mutant *Anabaena* sp. PCC7120 cells.

Transaldolase and Glucose-6-phosphate Dehydrogenase (G6PDH) Assays

Growth of *Anabaena* sp. PCC7120 wild-type and mutant cells followed measurement of optical density at 750 nm (24). Cells grown for 10 days were harvested and supernatants were prepared as enzyme solution,

Table 1. Bacterial strains and plasmids.

Bacterial Strain or Plasmid	Characteristics	Reference
Bacterial Strains		
Anabaena PCC7120	wild-type	14
HK28	*Anabaena* sp. PCC7120 *tal*: :pAUG401, Suc^s Sm^r/Spc^r Em^r Cm^r	This study
HK29	*Anabaena* sp. PCC7120 *tal*: :Ω Suc^r Sm^r/Spc^r	This study
E. coli AEE101	*pro met* carrying conjugative plasmid RP4	D. Hodgson (per. comm.)
E. coli HB101	F- Δ(*gpt-pro*A)62 *leu sup*E44 *ara*14 *gal*K2 *lac*Y1 Δ(*mcr*C-*mrr*) *rps*L20 (*str*)*xyl*-5 *mtl*-1 *rec*A13	15
E. coli MC1061	*ara*D139, Δ(*ara-leu*)7697, Δ(*lac*)X74, *gal*U$^-$ *gal*K$^-$, *hsd*R$^-$ *rps*L	16
E. coli TG1	K12, Δ(*lac pro*) *sup*E*thi*-1*hsd*D5 F'*tra*D36*pro*A$^+$B$^+$ *lac*I^q,ZΔM15	17
Plasmids		
pBR325	Cloning vector containing *oriT*; Cm^r Ap^r Tc^r	18
pRL271	Non-replicating cyanobacterial vector containing *sacB*, Cm^r Em^r	19
pRL6	Shuttle vector Cm^r Nm^r	20
pRL528	Helper plasmid for mobilisation used in triparental mating, Cm^r	20
RP-4	Conjugative plasmid; Ap^r Km^r Tc^r	21
pHP45Ω	Cloning vector containing interposon Ω Ap^r Sm^r Spc^r	22
pAG75	pBR325: 7 kb *Hin*dIII cut *Anabaena* sp. PCC7120 chromosomal DNA; Ap^r Cm^r Tc^r	9
pAUG20	pAG75: Ω into *Hpa*I site of pAG75; Ap^r Cm^r Tc^r Sm^r Spc^r	This study
pAUG401	*Nru*I digested pRL271: 5.2 kb *Eco*RI digested pAUG20	This study

according to the method described by Schaeffer and Stanier (25). Transaldolase assays were carried out as previously described (26). G6PDH assays were performed according to the method of Schaeffer and Stanier (25). One unit of enzyme activity was defined as the formation of 1 µmol of product in 1 min. Specific activity was expressed as units mg^{-1} of protein. The quantity of protein was determined using the Bio-Rad protein assay kit, according to the manufacturer's instructions.

Results and Discussion

Interruption of the *tal* Gene By Insertion of the Ω Fragment in the Gene

We previously cloned and sequenced the *tal* gene of *Anabaena* sp. PCC7120 (9). The gene was also cloned and sequenced from *Nostoc* sp. ATCC29133 (10). Later, genomic research showed that cyanobacterial strains have 2 copies of *tal* genes (11,12). Despite relatively poor database homologies, these genes were identified on the basis of the similarity of their translation products to transaldolases from other organisms, such as *E. coli* (27, 28) and *Saccharomyces cerevisiae* (29); therefore, a mutagenesis study on the *tal* gene of *Anabaena* sp. PCC7120 was carried out to confirm the identity of the *tal* gene and to characterise the function of transaldolase in this organism, as well as the effect of the *tal* gene on the function of the downstream *zwf* gene.

The interposon Ω with transcriptional termination signals and translation stop codons, as well as the resistance gene for streptomycin and spectinomycin (30) were used to inactivate the *tal* gene in the *Anabaena* sp. PCC7120 genome. The clone pAG75 contained the *tal* gene of *Anabaena* sp. PCC7120 between 2 other genes, encoding 2 OPP pathway enzymes: fructose-1,6-bisphosphate and glucose-6-phosphate dehydrogenase (G6PDH) (Figure 1). An *Hpa*I site occurs in the middle of the *tal* gene and this site was used to insert the interposon in the gene to yield pAUG20, containing an interrupted copy of the *tal* gene of *Anabaena* sp. PCC7120. This clone was selected against streptomycin

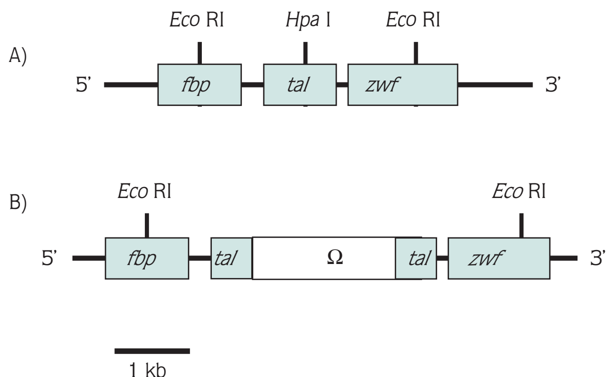

Figure 1. Restriction map of the clone (pAUG20). A 7.5-kb *Hin*dIII fragment of the *zwf* region of *Anabaena* sp. PCC7120 is inserted into the vector, pBR325. Then, 2.0-kb interposon *omega* is inserted into the *tal* gene at the *Hpa*I site. The bar indicates the size of insert and interposon.

and spectinomycin, resistance to which was provided by the Ω.

Introduction of the *tal* Mutation into *Anabaena* sp. PCC7120 Cell and Selection of Mutants

Conjugation is the most reliable way to introduce DNA into filamentous cyanobacteria (20,31), although some reports describe electrotransformation of strains of *Anabaena* sp. and *Nostoc* sp. (32,25). Versatile vector systems such as pRL271 were developed for filamentous cyanobacteria, particularly for *Anabaena* sp. PCC7120 and some *Nostoc* sp. strains (34). These vectors contain the *sacB* gene of the Gram-positive bacterium, *Bacillus subtilis* (35). The product of *sacB* is lethal for Gram-negative bacteria, including *Anabaena* sp. PCC7120, and this provides a positive selection for double recombinants. Plasmid pRL271 provides an *oriT* region and a *sacB* gene, as well as 2 antibiotic resistance genes for chloramphenicol and erythromycin, but lacks a cyanobacterial replicon (19,34) (Table 1); therefore, the selection for antibiotic resistance selects for recombination into the chromosome. Plasmid pAUG20 was digested with *Eco*RI to yield a 5.2-kb fragment containing the *tal* mutation. The cohesive ends of the fragment produced by *Eco*RI were filled and ligated into the *Nru*I site of plasmid pRL271 to yield pAUG401. This plasmid contained an insertion carrying the *tal* mutation, the interposon Ω in the middle of the *tal* gene, the *sacB* gene, and genes for resistance to 4 antibiotics (chloramphenicol and erythromycin resistance genes from the vector pRL271, and streptomycin and spectinomycin resistance genes from the interposon Ω). This plasmid also possesses an *oriT* region, which is necessary for conjugal transfer of the plasmid.

After allowing plasmid pAUG401 to integrate into the *Anabaena* sp. PCC7120 chromosome, exconjugant cells were selected with streptomycin and spectinomycin (2.5 μg ml^{-1} in solid medium and 1.5 μg ml^{-1} in liquid medium). The cells resistant to these antibiotics should contain either plasmid pAUG401 integrated into the chromosome by a single recombination, or the interposon in the *tal* gene and surrounding DNA exchanged by double recombination at the homologous regions, both upstream and downstream of the *Hpa*I site of the *tal* gene.

After growth on antibiotic-containing solid medium, colonies were transferred onto fresh solid medium supplemented with sucrose, in addition to streptomycin and spectinomycin. These colonies may be either single recombinants or double recombinants. Colonies carrying single recombinant chromosomes should not be able to survive on this medium because of the expression of *sacB*, which is lethal to *Anabaena* sp. PCC7120. Only 2 of the initial 20 colonies were able to grow on sucrose-supplemented medium. A representative sucrose-sensitive colony (HK28) and a sucrose-resistant colony (HK29) were transferred into liquid BG11 medium. To segregate the wild-type chromosomes from the double and single recombinant strains (HK28 and HK29), filaments were fragmented and the cells were successively grown in antibiotic-containing liquid medium.

Characterisation of the Mutants HK28 and HK29

Southern blot analysis and enzyme assays for transaldolase and G6PDH were performed, and growth properties were determined for the characterisation of the single and double recombinant strains. Southern blot analysis showed that the Ω fragment was successfully inserted in the *tal* gene (Figure 2). While a 3.2-kb *Eco*RI fragment contains the wild-type *tal* gene, the size of this fragment increased to 5.2 kb in the double recombinant strain, HK29. This size corresponds to the 3.2-kb wild-type chromosomal DNA plus the 2.0-kb Ω fragment. All the double recombinants analysed carry a 5.2-kb band, indicating the insertion of the mutant copy of the *tal* gene into the chromosome. However, 2 of the double recombinants also contain wild-type copies of the *tal* gene; therefore, 2 double recombinants possess only a mutant copy of the *tal* gene. These double recombinants were thus taken as *tal* mutant strains. After confirming

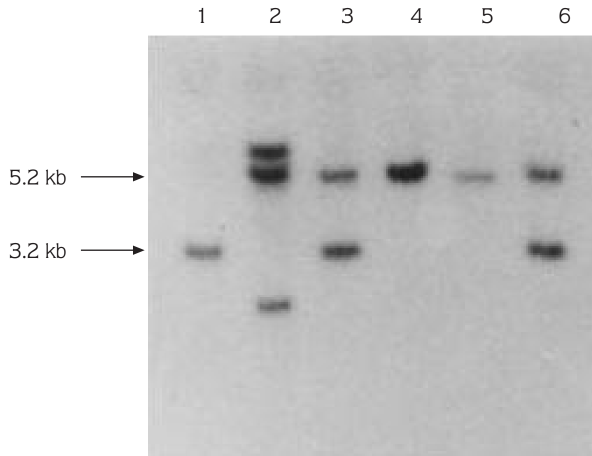

Figure 2. Southern blot analysis of the wild-type, and single and double recombinants. Chromosomal DNA was digested with *Eco*RI, producing a 3.2-kb fragment that contained *tal* in the wild-type chromosome. This size, theoretically, rises to 5.2 kb in the chromosomes of double recombinant cells due to insertion of a 2.0-kb interposon into the *tal* gene. Lane 1 is the wild-type, lane 2 is the single recombinant strain (HK28), and lines 3-6 are the double recombinant *tal* mutant strain (HK29). All double recombinants carry mutated copies of *tal* (5.2 kb), but 2 of them (lanes 3 and 6) also possess the wild-type copy of the gene. Single recombinant cells give various bands because of integration of the clone (pAUG401) into the chromosome (lane 2).

the nature of the single and double recombinant cells they were further analysed for transaldolase activity, and for growth in the presence and absence of combined nitrogen.

There were no significant differences between the growth of the double and single recombinants, and the wild-type in the presence of combined nitrogen (Figure 3A); however, the double and single recombinant cells reached the stationary phase relatively faster than the wild-type in the absence of combined nitrogen, both showing a similar growth pattern (Figure 3B). Transaldolase assays showed that the activity in the double recombinant was similar to that of the wild-type and that 72.4% of the activity was retained in the single recombinant cells (Table 2).

The results of the transaldolase assays and measurements of the rate of growth of the wild-type and mutant cells suggested that the *Anabaena* sp. PCC7120 genome carries more than 1 gene that encodes transaldolase; therefore, full transaldolase activity in the *tal* mutant cells and growth in the absence of combined nitrogen may be because the other copy of the *tal* gene in *Anabaena* sp. PCC7120 genome complements transaldolase activity. However, the early entry of the single and double recombinant cells into the stationary phase cannot be explained by the results of the transaldolase assays. This may be due to results of the polar nature of the interposon mutation on the transcription of the genes in this region of the genome.

In all, 4 genes of *Nostoc* sp. ATCC29133 were reported to be arranged in a single operon: *fbp, tal, zwf*, and *opcA* (36). These genes could be cotranscribed either in groups or individually by more than 1 promoter, producing more transcripts of the genes located at the downstream part of the operon, *zwf*, and *opcA* in this operon (13). Being a close relative of *Nostoc* sp.

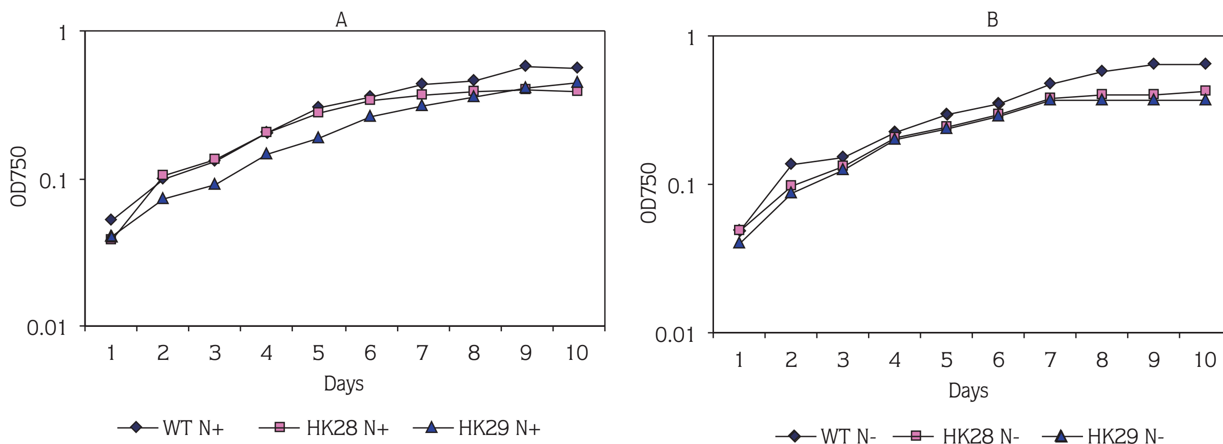

Figure 3. Growth rates of the wild-type, the single recombinant strain (HK28), and the double recombinant (HK29). (A): Growth rates in the presence of combined nitrogen. (B): Growth rates in the absence of combined nitrogen (N+: combined nitrogen is present; N-: combined nitrogen is absent).

Table 2. Transaldolase and G6PDH activity in the wild-type single recombinant strain, HK28, and in the double recombinant strain, HK29. Each value is the average of 3 independent measurements and activity is expressed as specific activity (units mg^{-1} of protein).

Strains	Transaldolase Activity (% WT activity)	G6PDH Activity (% WT activity)
Wild-type	82.9 (100%)	136.6 (100%)
HK28	60.1 (72.4%)	33.7 (24.6%)
HK29	91.1 (109.9%)	46.8 (34.1%)

ATCC29133, *Anabaena* sp. PCC7120 was tested to determine if such an arrangement existed in the *zwf* region. The genes are arranged as *fbp-tal-zwf* in this region of *Anabaena* sp. PCC7120 (9, 12). If these genes are arranged in a single operon, the insertion of the interposon Ω in the *tal* gene would result in the complete cessation of transcription downstream of the insertion point, or full transcription, unless these were alternative promoters located downstream of the insertion point. G6PDH assays implied that the genes in the *zwf* region of *Anabaena* sp. PCC7120 were arranged in a single operon transcribed by more than 1 promoter. This was judged by the retention of only 34% of G6PDH activity in the double recombinant (HK29) (Table 2). This low level of G6PDH activity also explains the early entry of the double and single recombinant cells into the stationary phase in the absence of combined nitrogen. Since G6PDH is an important supplier of reducing power and energy to nitrogenase through the OPP cycle, the cells that exhibit a lower level of G6PDH activity cannot grow at the same rate as the wild-type in the absence of combined nitrogen. This low level of G6PDH activity would limit the rate of nitrogen fixation and the rate of growth.

Consequently, the results of the transaldolase assays of mutant cells indicate that mutation of 1 or 2 copies of the *tal* gene does not affect transaldolase activity. Accordingly, the mutant was grown similarly as wild-type under continuous light. This result is consistent with the *talB* mutant of *E. coli* growing normally in glucose-minimal medium. Since it exhibits no transaldolase activity, the *E. coli talB* mutant owes its normal growth to the flexibility of its central metabolism (7). In contrast, *Anabaena* sp PCC7120 transaldolase activity was not affected by *tal* mutation. This may have been due to the complementation of transaldolase activity by the other copy of the gene. On the other hand, the lower level of G6PDH activity in the *tal* mutant cells implies that the genes in the *zwf* region of *Anabaena* sp. PCC7120 are arranged in a single operon, similar to that in *Nostoc* sp. ATCC29133 (13). This low level of G6PDH activity may affect the growth rate in the absence of combined nitrogen.

In conclusion, a mutation of the *tal* gene located in the *zwf* region did not affect transaldolase activity, producing no significant difference in the growth rate with combined nitrogen. Relatively faster entry into the stationary phase without combined nitrogen may have been affected by low-level G6PDH activity due to the polar effect of the *tal* mutation on the transcription of the *zwf* gene.

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