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Systematic study of *acuABC* operon in *Bacillus subtilis* 168

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Abstract *Bacillus subtilis* grown in media containing glycolytic carbon sources secretes pyruvate, organic acids and acetoin. Acetoin can be reused by *Bacillus* when other carbon sources are exhausted from growth media. *acuABC* and *acoABCL* operons are two proposed pathways for acetoin catabolism. However the mode of involvement of *acuABC* is not well understood yet. The work presented here focuses on the comparative study of *acuABC* and *acoABCL* operon. We have observed an accumulation of acetoin in a 168acuA- strain upto the late stationary phase of growth. Promoter expression studies in acetoin and other carbon source suggest that *acuABC* operon had a direct role in acetoin catabolism.

Keywords: *Bacillus*, Acetoin, Acetoin catabolism

1. Introduction

Acetoin is a four carbon acetohydroxy compound that is synthesized in all simple bacteria, plants and animals, as the by-product of branched chain amino acid synthesis. Organisms such as *Klebsiella* and *Bacillus* synthesize acetoin during late exponential phase of the growth by *alsSD* operon. The *alsSD* operon consists of *alsS* and *alsD* enzyme units that code for acetolactate synthase and acetolactate decarboxylase respectively. In *Bacillus*, acetoin is used as a secondary carbon source during sporulation. The possible routes for acetoin catabolism are by direct oxidative cleavage, butanediol pathway, *acoABCL* operon or *acuABC* operon. The most studied pathway for acetoin catabolism is *acoABCL* (Huang *et al.*, 1999). Grundy and coworkers have observed (1993) that deletion of *acuA* leads to decrease in the efficiency of growth on acetoin as sole carbon source. Further studies show that it has sequence similarities with *atoABC* operon of *E. coli* (Leipe and Landsman, 1997). This operon is responsible for metabolism of acetate. In 2006, Gardner and coworkers have shown the interaction of *acuA* protein with acetate and suggested that involvement of *acuABC* operon in acetoin catabolism is indirect. The role of *acuABC* operon in acetoin catabolism is therefore not yet fully understood.

To compare the efficiency of catabolism of these two operons, translational fusion strains are constructed by cloning upstream regions for *acuA* and *acoA* promoter. These strains were grown in various conditions of carbon sources and reporter (β-galactosidase) activity was measured by Miller's method.

2. Materials and methods

2.1 Strains, plasmids and cloning procedures

The strains used in this study are listed in Table 1.Transformation of *Escherichia coli* DH5α cells was performed by calcium chloride method as described by Sambrook *et al.,* 1989. Transformation of *B. subtilis* cells for the integration of selected DNA sequences into the chromosome was performed as described by Hoch, 1991.

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The conditions used for electroporation was as follow: 2500 V/25 μF/200 Ω/2-mm electrode gap with 1 μg DNA in 100 μl cell solution containing 30% (w/v) polyethylene glycol.

The cells were grown in Luria-Bertani Broth to an optical density (OD 600 nm) of 1.5 and incubated at 4 °C for 1 h before electroporation. Plasmid transformation in *Bacillus* cells was performed by Calcium chloride method as described by Hoch, 1991.

Table 1 Strains

Strain	Relevant genotype	Reference
Escherichia coli DH5α	*supE*44Δ*lacU*169(φ80*lacZ* ΔM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Laboratory stock
Bacillus Subtilis 168	*trpC2*	Laboratory stock
Bacillus subtilis acuA-	*trpC2, acuA::cat*	This work
Bacillus subtilis AE101	168 containing pRB381 plasmid	This work
Bacillus subtilis AE102	168 containing pRB381pacua plasmid	This work
Bacillus subtilis AE103	168 containing pRB381pacoa plasmid	This work

To delete the *acuA* gene on the *B. subtilis* chromosome, the plasmid pBSKacuAcat was constructed. Homologous sequences upstream and downstream of *acuA* were amplified with the primer pair PS03/04 (Table 3) and cloned into pBSK. Subsequently, the chloramphenicol resistance gene was isolated from pBSKcam by restriction digestion by *EcoRV* and then subcloned in pBSKacuA at *Nru1* site to construct plasmid pBSKacuAcat. This plasmid was used for the transformation of strain *Bacillus subtilis* 168, resulting in the *acuA* deletion strain 168acuA-. To confirm the replacement of wild type copy of acuA PCR analysis was performed by primers PS21/22. A 1.1 Kb increase in size of amplification was observed in 168acuA- in comparison to *B. subtilis* 168. All the plasmids used in study are listed in table 2.

Furthermore, the upstream regions of *acuA* and *acoA* gene were cloned on plasmid pRB381 after amplification with the primer pair PS25/26 and PS27/28 respectively, resulting in pRB381acuA and pRB381acoA respectively. *B. subtilis* 168 was transformed with pRB381PacuA and pRB381PacoA, resulting in AE102 and AE103 strains respectively.

Table 2 Plasmids

Plasmid	Relevant genotype	Reference
pBSK	Phagemid cloning vector for *E. coli*, Apr	Laboratory stock
pBSKacuA	pBSKcontaing ~1100 bp of *acuA* gene cloned at *XbaI* and *BamHI* sites	This work
pBSKcam	pBSK vector containing chloramphenicol acetyl transferase gene (CAT)	Laboratory stock
pBSKacuAcat	CAT gene cloned at *NruI* site of pBSKacuA	This work
pRB381	*E. coli-Bacillus subtilis* shuttle vector for translational fusion with the β-galactosidase gene	Laboratory stock
pRB381Pacua	pRB381 vector containing 800 bp of *acuA* gene cloned at *Pst1* and *BamHI* site	This work
pRB381Pacoa	pRB381 vector containing 750 bp of *acoA* gene cloned at *Pst1* and *BamHI* site	This work

2.2 Media and cultivation

The *B. subtilis* and *E. coli* cultures were grown in LB (Luria Bertani, Miller) medium (Hi-Media) (10 g/L casein enzymatic hydrolysate, 5 g/L yeast extract and 10 g/L sodium chloride, supplemented with appropriate antibiotics) at 37^0C under shaking conditions. The defined medium used for growing *Bacillus* strains was TSS medium; TSS (0.05 M Tris [pH7.5], 40 g each of $FeCl_3.6H_20$ and trisodium citrate dihydrate

per ml, 2.5 mM K_2HPO_4, 0.02% $MgSO_4.7H_20$, 0.2% glutamine, 0.5% glucose or 0.2% trisodium citrate dihydrate liquid medium. When necessary, TSS was supplemented with amino acids (to 0.004%). 1.5 % of agar was added for preparation of solid medium. To study the promoter activity strains were grown in Penassay broth and 0.5% of carbon (glucose/acetoin) source was added to study the effect on expression of promoters.

Table 3 Primers

Primer name	***Sequence 5'-3'***
PS03	CGCTCTAGAAATAAAGCTCGGGTGATCTCGG
PS04	CGCGGATCCCCCCTTTGTCAGTGTCAGTTAATAC
PS21	ATGAACTTGAAAGCGTTACCAGCAATAGAG
PS22	ATGAAAAAACTTGTGTTTGGCTTGCTTGCC
PS25	CGAGATCTCTGCAGTATGATCCCCCTCTATTGC
PS26	CTTAGGATCCCTCTGGAGAGACAGGCCCTTCTAT
PS27	CGAGATCTCTGCAGATTGTCAAAGGCCGGGTGAT
PS28	CTTAGGATCCAAATGGGGTAGCTTCGCCATAGCCGT

3. Results and Discussions

Bacillus subtilis 168 grown in a medium containing glucose or any other carbon source that can be metabolized through glycolytic route produces acetoin in the late exponential phase of growth. Acetoin undergoes further reaction by the acetoin dehydrogenase enzyme complex and is converted to acetyl coA and acetaldehyde. In our experiments, the effect of deletion of *acuA* on growth and acetoin accumulation was studied in minimal medium. Furthermore, the *acuA* promoter upstream region was cloned in the translational fusion vector having LacZ as reporter gene. This promoter activity was compared with preexisting operon *acoABCL*. The *acuA* and *acoA* promoter activities were compared on various levels of glucose and acetoin.

3.1 Effect of deletion of *acuA* on growth

Bacillus subtilis acuA- was grown in TSS medium using 0.5 % of acetoin and glucose as a sole carbon source. OD_{600} was measured as a parameter of growth. The growth was compared for *Bacillus subtilis* 168 and *Bacillus subtilis acuA-* on glucose (Figure 1a) and on acetoin (Figure 1b) as a sole carbon source. Maximum OD_{600} of *Bacillus subtilis* 168 and *acuA-* on glucose were both observed to be 4.9; this shows that the 168acuA-strain is not defective in growth on glucose. A significant difference in growth of *Bacillus subtilis*168 and 168acuA- was however observed when acetoin was used as a sole carbon source, with OD_{600} for *Bacillus subtilis*168 and 168acuA- being 4.2 and 2 respectively after 42 hr of the growth. This shows that disruption of *acuA-* leads to defect of growth when acetoin is used as a sole carbon source.

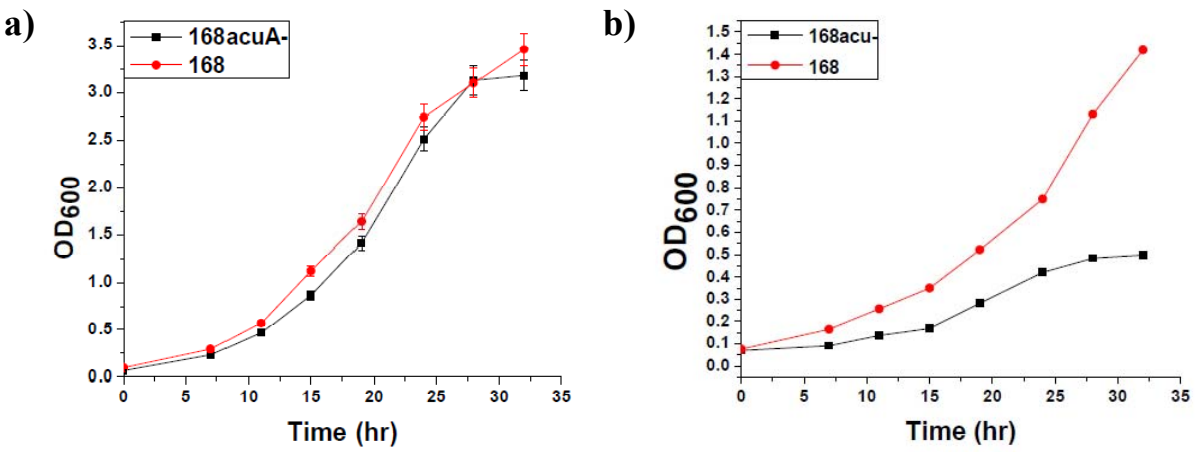

Figure 1.Comparison of growth of 168 and 168acuA- when grown in TSS medium containing (a) Glucose (b) Acetoin as sole carbon source.

3.2 Effect of deletion of *acuA* on acetoin accumulation

To study the effect of deletion of *acuA* gene of acetoin catabolism; *Bacillus subtilis* 168 and 168acuA- were grown in TSS medium by providing acetoin as sole carbon source. We have observed a higher amount of acetoin remains in the medium at the late stationary phase of growth.

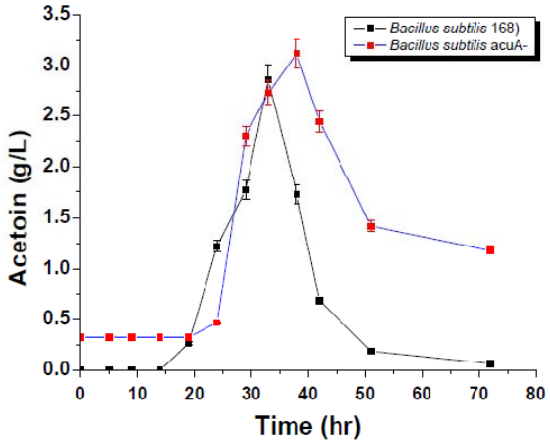

Figure 2. Effect of deletion of *acuA* on acetoin accumulation when grown in TSS medium containing 0.5 % acetoin as sole carbon source.

3.3 Result of addition of glucose on expression of *acuA* and *acoA*

Bacillus subtilis AE102 and 103 strains were inoculated in Penassay broth and 0.5% of glucose was added to a 5 hr old (exponential phase) culture. Aliquots were taken at desired time points and β-galactosidase activity was measured for strains AE101, AE102 and AE03. We have not observed any significant growth defect while growing in Penassay broth on providing the appropriate antibiotic. All the strains were repeatedly grown for 10-15 generations and were observed to be stable hosts for the plasmids pRB381, Pacu, Paco. We have not observed any activity for the promoter; probably due to catabolite repression in presence of glucose. No effect on growth was observed as a consequence of transformation with these reporter gene-bearing plasmids.

3.4 Result of addition of acetoin on expression of *acuA* and *acoA*

Experiments were performed as described in section 3.3. A 0.5% acetoin was added to the 5hr exponential phase culture. All strains were grown on Penassay broth. To test the possibility that acetoin induces reporter gene expression under the control of *acuA* or *acoA* promoters, 0.5% acetoin was added at the 5 hour time point and β- galactosidase activity was measured. The addition of acetoin did not affect growth.

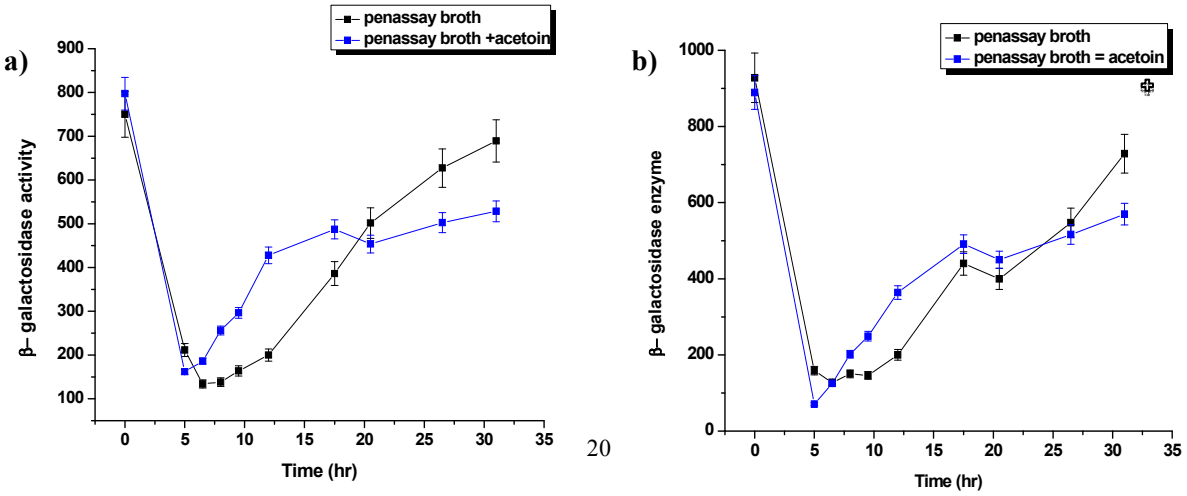

Figure 3.The effect of addition of acetoin on the expression of (a) *acuA* (b) *acoA* promoters in Penassay broth.

We have observed that addition of acetoin causes an induction in expression of *acuA* and *acoA* promoters. The β- galactosidase activities in glucose and acetoin inducing conditions were comparable for *acuA* and *acoA* promoters; this suggests that *acuABC* and *acoABCL* promoters are equally strong.

4. Conclusions

The results of these experiments show that deletion of the *acuA* gene only has an effect on growth when the strains were grown on acetoin as sole carbon source. It suggests that function of *acuA* is not required for vital growth of *Bacillus*. However a substantial growth difference was observed in *Bacillus subtilis*168 and 168acuA- when grown on acetoin. A 50% lesser acetoin was degraded in case of 168acuA-. This strongly suggests a role for *acuA* in acetoin catabolism. Further to investigate type of (direct or indirect) involvement of *acuA* in acetoin metabolism; translation fusion strains AE102 and AE103 were constructed for *acuA* and *acoA* promoters respectively. A negligible β- galactosidase activity was observed for AE102 and AE03 strains. The probable reason for this might be due to catabolite control of these operons. When induction was performed by addition of acetoin; an early expression of promoter of these genes was observed. This strengthens the hypothesis of direct involvement of *acuABC* operon in acetoin catabolism.

5. Acknowledgements

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6. References

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