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AcpA, a member of the GPR1/FUN34/YaaH membrane protein family, is essential for acetate permease activity in the hyphal fungus *Aspergillus nidulans*

Xavier Robellet¹, Michel Flipphi², Sylvine Pégot¹, Andrew P. MacCabe²
and Christian Vélot^{1*}

From the ¹Institut de Génétique et Microbiologie, Unité Mixte de Recherche 8621, CNRS - Université Paris-Sud XI, Centre Scientifique d'Orsay, Bâtiment 360, F-91405 Orsay Cedex, France,

and the ²Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Apartado de Correos 73, 46100 Burjassot, Valencia, Spain

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Address correspondence to: Christian Vélot, IGM, Centre Scientifique d'Orsay, Bât. 360, 91405 Orsay Cedex. E-mail: christian.velot@igmors.u-psud.fr, Tel.: (33) 1 69 15 82 95, Fax: (33) 1 69 15 63 34.

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ABSTRACT

In a previous study, *alcS*, a gene of the *Aspergillus nidulans alc* cluster, was shown to encode a protein that belongs to the GPR1/FUN34/YaaH membrane protein family. BLAST screening of the *A. nidulans* genome data identified additional genes encoding hypothetical proteins that could belong to this family. In this study we report the functional characterization of one of them, *AN5226*. Its expression is induced by ethanol and ethylacetate (two inducers of the *alc* genes) and is mediated by the specific transcriptional activator of genes of the acetate-utilization pathway FacB. Growth of a null mutant ($\Delta AN5226$) is notably affected when acetate is used as sole carbon source at low concentration and in a high pH medium, *i.e.* when protonated acetate, the form that can enter the cell by passive diffusion, is present in low amounts. Consistently, expression of *AN5226* is also induced by acetate, but only when the latter is present at low concentrations. ^{14}C -Acetate uptake experiments using germinating conidia demonstrate an essential role for *AN5226* in mediated acetate transport. To our knowledge this report is the first to provide evidence for the identification of an acetate transporter in filamentous fungi. We have designated *AN5226* as *acpA* (for acetate permease).

INTRODUCTION

Like other filamentous fungi, *Aspergillus nidulans* is able to grow on two-carbon compounds, such as acetate and ethanol, as sole carbon sources. The ethanol utilization pathway of *A. nidulans* (termed the *alc* system) requires two structural genes, *alcA* and *aldA*. These encode the enzymes alcohol dehydrogenase and aldehyde dehydrogenase, respectively, which catalyse the conversion of ethanol into acetate via acetaldehyde. Induction of *alcA* and *aldA* expression is controlled by a regulatory gene, *alcR*, encoding the pathway-specific autoregulated transcriptional activator [1]. To enter mainstream metabolism, acetate is further converted into acetyl-CoA by acetyl-CoA synthetase, encoded by *facA* [2, 3]. Acetyl-CoA is either catabolized by the Krebs tricarboxylic acid (TCA) cycle in the mitochondrion or assimilated by the glyoxylate cycle, an anaplerotic version of the TCA cycle that is absolutely required for the utilization of two-carbon compounds when they are present as sole carbon sources. The glyoxylate pathway, present in bacteria, plants and fungi, enables the synthesis of C4 dicarboxylic acids from acetyl-CoA units, bypassing the two decarboxylation steps in the TCA cycle. The C4 metabolites so produced can be used for energy production, gluconeogenesis and other biosynthetic pathways. In *A. nidulans*, the two enzymatic activities specific to the glyoxylate cycle, isocitrate lyase and malate synthase, are encoded by *acuD* [4] and *acuE* [2, 3], respectively. Unlike *alcA*, *aldA* and *alcR*, the *acuD*, *acuE* and *facA* genes are under the control of FacB [3, 5], the acetate utilization pathway-specific transcriptional activator.

alcR and *alcA* are clustered on the left arm of chromosome VII together with three other genes, *alcO*, *alcM* and *alcS*, that are subject to strict AlcR control [6]. However, their functions remain unknown. In a previous study [7] we showed that *alcS* encodes a plasma membrane protein belonging to the GPR1/FUN34/YaaH family which includes among others, Gpr1p of *Yarrowia lipolytica* [8] and Ady2p and Fun34p of *Saccharomyces cerevisiae* [9, 10]. While no clear biochemical function has yet been established for any protein in this family (Transporter Classification system: TC-9.B.33), phenotypic effects have been described in yeast species for *gpr1* and *ady2* mutants in relation to the utilization of acetate as the growth substrate. In *S. cerevisiae*, *ADY2* expression (as well as that of *FUN34*) is elevated in liquid culture upon a shift from glucose medium to the non-fermentative growth substrate acetate. Deletion of *ADY2* results in the loss of mediated acetate uptake, implying that this gene is necessary for the expression of an acetate permease [11]. In *Y. lipolytica*, deletion of the *GPR1* gene leads to a prolonged lag phase in growth on acetate [12]. Moreover, a recent study shows that certain mutations in *ADY2* and *FUN34* likewise induce acetic acid

sensitivity in *S. cerevisiae* [13]. By contrast, *A. nidulans alcS*-deleted mutants do not display any obvious phenotype (on ethanol nor on acetate), suggesting that some other gene/genes might be able to compensate the absence of *alcS* under the conditions tested. A BLAST search of the *A. nidulans* genome [14] led to the identification of two novel genes that are induced by ethanol and ethylacetate (inducers of the *alc* system) and that could encode other members of the GPR1/FUN34/yaaH family, AN5226 and AN8390 [7].

Here we report the functional characterization of AN5226, now designated AcpA (for acetate permease A). An *acpA* gene deletion mutant shows notably reduced growth when acetate is used as a sole carbon source at low concentration and in a high pH medium. ¹⁴C-Acetate uptake experiments using germinating conidia lead us to identify AcpA as an essential component of mediated acetate transport.

EXPERIMENTAL

Strains, media, general growth conditions: The *A. nidulans* strains used in this study are listed in Table 1. Genotypic annotations have been described previously [15]. Media composition, supplements and basic growth conditions were as described by Cove [16]. Nitrogen sources were generally added to a final concentration of 10 mM nitrogen, *i.e.* diammonium tartrate and urea were each used at 5 mM. Carbon sources were added at either 0.1%, 0.5% or 1% (w/v or v/v) as specified.

General molecular techniques, classical genetics, transformation: Standard molecular techniques were as described in Sambrook and Russell [17]. *Aspergillus* genomic DNA utilized for PCR reactions and in Southern blot analyses was isolated according to Specht et al. [18]. PCR products were purified using the High Pure PCR Product Purification Kit (Roche). Auxotrophic markers were exchanged and mutant alleles were combined through meiotic recombination employing conventional genetic techniques [19]. Protoplast generation and transformation of *A. nidulans* with PCR-amplified DNA were performed essentially as described by Tilburn et al. [20].

Structure of the *acpA* gene: Features of the *acpA* gene structure (intron positions, transcription start- and end-points) were revealed upon sequencing of cDNA clones obtained upon rapid amplification of cDNA ends (RACE) with a sample of total RNA. The RACE of *acpA* was performed with RNA isolated (according to the method described below) from a $\Delta alcS$ strain (CV171) induced with ethylacetate, utilizing a number of gene-specific oligonucleotide primers (Table 2). The GeneRacer kit from Invitrogen was used for the 5' RACE and the 5'/3' RACE kit 2nd generation from Roche was used for the 3' RACE.

Construction of the *acpA::panB* disruption cassette: A gene replacement strategy, based on the principle of double joint PCR [21], was designed to obtain *acpA* complete loss-of-function mutants. The 5' and 3' flanking sequences of *acpA* were amplified using the oligonucleotide couples 5226-5'-anti/5226-5'-pto3'-sht and 5226-3'sens/5226-3'-pto5'-sht (Table 2). The oligonucleotides 5226-5'-pto3'-sht and 5226-3'-pto5'-sht have 28 and 30 base-long homologies with the 3' and 5' non-coding regions of the ketopantoate hydroxymethyl transferase (*panB*) gene [22], respectively. Genomic DNA from strain BF107 was used as the template for PCR amplifications of the 5' and 3' *acpA* flanking regions. The *panB* gene was amplified using the oligonucleotide couple pto3'-5226-5'sht/pto5'-5226-3'sht (Table 2). These oligonucleotides have 28-29 base-long homology with the 5' and 3' non-coding regions of the *acpA* gene. The plasmid pJVF089 carrying a functional *A. nidulans panB* gene [23] was used as the template for this PCR. After gel purification, equimolar amounts of the three fragments obtained were fused by means of a PCR without oligonucleotide primers under the following conditions: 5 min at 94°C; 15 cycles of (45 s at 94°C, 2 min at 58°C and 8 min at 68°C) and 15 min post-polymerisation with the Expand Long Template PCR System (Roche). Subsequently, the oligonucleotide couple 5226-5'anti/5226-3'sens were added and the same cycling program was executed except that the number of cycles was increased to 25. Finally, a PCR was performed with nested oligonucleotides, Nes5226-3'sens and Nes5226-5'anti (Table 2), to generate the *acpA::panB* disruption cassette (~6.5 kb).

Generation of the null $\Delta acpA$ and the double $\Delta acpA \Delta alcS$ mutants: To generate the *acpA* knock-out mutant, 3 μ g of the 6.5-kb *acpA* disruption cassette (see above) was used to transform the D-pantothenic acid-requiring *panB100* strain CV164 and the $\Delta alcS$ *panB100* strain CV171 (Table 1). Disruption of the *acpA* gene in a number of pantothenate prototrophic transformants was checked by Southern analysis. The resulting null $\Delta acpA$ and the double $\Delta acpA \Delta alcS$ mutant strains used in this study were termed CV260 and CV253 (Table 1). CV164 and CV171 were also transformed with the 2.5-kb *panB* fragment alone to generate the control strains CV312 and CV313 (*i.e.*, pantothenate prototrophic transformants carrying a wild-type *acpA* gene – see Table 1).

Transcript analysis: Mycelia for transcript analysis were grown for 24 h in appropriately supplemented minimal medium with lactose (3% w/v) as the carbon source and urea as the nitrogen source [24]. Media were buffered with 100 mM sodium phosphate pH 6.8 added to cooled autoclaved medium from filter-sterilized stocks. Induction was achieved by the addition of the inducers to the concentrations indicated and cultures were harvested after 2.5 h of further incubation. Total RNA was isolated from mycelia as described previously [25].

Northern analysis was carried out with 10 µg samples of glyoxal-treated total RNA essentially as described by Flipphi et al. [25]. DNA probes were labelled with ^{32}P dCTP using the MegaprimeTM DNA Labelling System (Amersham Biosciences). Hybridisation signals were revealed using a PhosphorImager (Molecular Dynamics). The γ -actin (*acnA*) gene served as a reference as it is considered to be expressed equally well in all strains and under all conditions tested.

Acetate uptake measurements: The isolation of germinating conidia and uptake measurements were performed essentially as previously described by MacCabe et al. [26]. Acetate uptake rates were measured by assaying the incorporation of [$1\text{-}^{14}\text{C}$] acetic acid (sodium salt, with a specific activity of 2.11 GBq/mmol; GE Healthcare) into conidia at various external acetate concentrations. Uptake was quenched by rapid addition of 1.5 ml ice-cold unlabelled 1 M acetate in minimal medium at pH 6.8. The nitrocellulose filters used to collect the germinating conidia were rinsed with 1.5 ml of the same acetate solution immediately prior to sample filtration. Acetate uptake rates were determined by linear regression of plots of the amounts of radiolabelled acetate taken up by a fixed number of 4-h old glucose germlings after short periods of incubation (*i.e.* 5, 30, 60, 90, 120 and 180 seconds) at 37°C in a fixed volume under continuous agitation. All data were analysed using SigmaPlot Version 8.02 (SSP Inc.)

RESULTS

Structure of the *acpA* gene and its putative translation product

Using RACE, the exact position of the *acpA* transcripts was mapped by analyzing the sequences of a number of *acpA*-specific cDNA clones generated from the same RNA sample obtained from ethylacetate-induced mycelium (see Experimental). The *acpA* open reading frame (ORF) and its flanking regions is shown in Fig. 1A. The complete DNA sequence determined is available under the GenBank accession number EF428327. Sequencing of the *acpA*-cDNA identified two intron sequences disrupting the coding region of the gene. The coding sequence formed upon their removal would encode a putative product of 298 amino acids. Consistent with the two *acpA* RNA products observed on northern blots [7], 5'-RACE experiments identified two 5' untranslated regions (5' UTR) of 398 and 247 bases in length. However, the two transcriptional initiation sites are 469 bases apart. This discrepancy is due to the absence of a 318 base sequence in the 5' UTR of the longer transcript running from position -339 to position -22 (relative to the presumed start codon) (Fig. 1A and 1B). This upstream intron sequence exhibits an unusual structure, in particular towards its 3' end, where

it is heavily enriched in adenine residues organized in consecutive polyA stretches located between a 216 base ORF found within the intron and the presumed intron lariat sequence (GCUGAC). The transcription initiation site of the shorter RNA product is located within this 5' UTR intron (Fig. 1A and 1B) hence the 5' UTR sequence is almost unique for each of the two transcripts, with the exception of the 21 bases immediately preceding the presumed start codon. To confirm the data obtained from these 5'-RACE experiments, we probed a northern blot made with total RNA isolated from ethylacetate-induced mycelia using probes specific for each of the *acpA* mRNA products (Fig. 1B). Whilst two transcripts are clearly produced (Fig. 1C), the absence of an upstream AUG in the 5' UTR of the longer mRNA make it likely that both transcripts give rise to the same translation product. The AcpA protein is a member of the GPR1/FUN34/YaaH membrane protein family (TC-9.B.33) [7]. When the deduced amino acid sequence of AcpA is subject to analysis using algorithms designed to address membrane protein topology it is predicted to have six unambiguous transmembrane domains flanked by two additional putative domains. The six family-characteristic membrane spanning segments are indicated in Fig. 1A. The two putative membrane spanning segments (not depicted in the Figure) comprise residues 13 to 33 and 268 to 288.

Deletion of acpA affects growth on acetate as sole carbon source at low concentration and in a high pH medium

Using a gene replacement strategy, a complete loss-of-function $\Delta acpA$ single mutant was constructed as well as a $\Delta acpA \Delta alcS$ double deletion mutant (see Experimental). The ability of these mutant strains to grow on different carbon sources was examined by plate testing. While no growth defects were observed on glucose, glycerol or ethanol, the ability of these mutants to utilize acetate appeared to be affected. Acetate is generally used at 0.5 % (w/v of the sodium salt) and *Aspergillus* media are normally set at pH 6.8, well above the pKa of acetic acid (4.75 at 25°C). [pKa is the pH value at which the dynamic pH-dependent equilibrium between the protonated (HA) and dissociated (A⁻) forms of acetate are present in equimolar concentration. At pH values below the pKa this equilibrium is shifted towards the protonated uncharged form whereas higher pH values result in predominance of the anion]. Plate growth was therefore tested not only at different acetate concentrations but also at different medium pH. As can be seen in Fig. 2, the $\Delta acpA$ mutants clearly manifest a growth defect at the low acetate concentration (0.1 %) which is more pronounced at high pH where the acid becomes increasingly limiting compared to the acetate anion. As could be expected,

re-introduction of the *acpA* gene into these gene-deleted mutants fully restored wild-type growth characteristics (data not shown).

Interestingly, growth on acetate was further reduced in the double $\Delta acpA \Delta alcS$ mutant compared to $\Delta acpA$, whereas a $\Delta alcS$ mutant grew just as well as the wild-type strain (Fig. 2). *A. nidulans* is able to utilize agar as a very poor carbon source in the absence of any other growth substrate (*i.e.* under conditions of carbon starvation), leading to colonies of low hyphal density and sparse sporulation [3]. In this regard, starvation growth of a $\Delta alcS$ mutant on minimal medium agar plates lacking an added carbon source is compromised compared to that of a wild-type strain or a single $\Delta acpA$ null mutant (data not shown). This defect in the $\Delta alcS$ mutant could therefore explain the more severe phenotype observed for the double $\Delta acpA \Delta alcS$ mutant on limiting acetate plates.

acpA is required for induction of the acetate-utilization pathway genes by limiting amounts of externally applied acetate

Considering the results presented above, we carried out northern blot experiments to test the ability of acetate to induce the expression of the genes required for acetate utilization, such as *acuE*, in the $\Delta acpA$ mutant when acetate is used at 0.1 % (*i.e.* 7.2 mM) in medium of pH 6.8 (Fig. 3A). At this pH the dissociated form (A^-) represents more than 99% of the acetate molecules. While *acuE* is strongly induced under these conditions in a wild-type strain, no induction was observed in the null $\Delta acpA$ mutant. Deletion of *acpA* thus results in a phenotypic effect similar to that originally described by Kujau et al. [8] for glyoxylate pathway regulator (*gpr*) mutations in *Y. lipolytica*, including among others *gpr1* mutations. To confirm that the lack of *acuE* induction is due to the inability of acetate to enter the cell in this mutant, we also tested the ability of ethanol and ethylacetate (at the same low concentration) to induce *acuE* expression in the $\Delta acpA$ strain. Induction of the acetate-utilization pathway genes by ethanol and ethylacetate is due to the production of acetate resulting from the intracellular catabolism of these carbon sources [24, 27] and hence does not involve any acetate uptake. No differences in the ethanol and ethylacetate induction patterns were observed between the wild-type and the $\Delta acpA$ mutant strains (Fig. 3B). Conversely, ethanol induction of *acuE* is absent in *alcR* mutants (data not shown) as these cannot catabolize ethanol into acetate [1]. These data support the hypothesis that AcpA is involved in the uptake of the dissociated form of acetate.

Expression of the acpA gene is induced by low concentrations of acetate

In a previous study we reported that *acpA* (AN5226) itself is not induced by acetate but rather expressed at a basal level similar to that observed on lactose [7]. However, in those experiments acetate was used at a concentration of 36 mM (0.5%) in non-pH stabilised medium. The present results (described above) indicate that *acpA* is required primarily when the protonated form of acetate (HA) is present at limiting concentrations that are insufficient for growth. We therefore analysed transcriptional expression of *acpA* in a wild-type strain grown at pH 6.8 in response to increasing concentrations of acetate (from 7.2 mM to 36 mM, *i.e.* from 0.1 to 0.5%) using *acuE* as a control. As shown in Fig. 4 *acpA* is clearly induced by low amounts of acetate, its level of expression apparently being inversely correlated to the acetate concentration; up to 28 mM, *acuE* induction appears to follow the opposite trend. This *acpA* transcription profile can account for the growth phenotypes shown in Fig. 2.

The dramatic drop in the *acuE* transcript level observed between 28 and 36 mM of acetate in medium phosphate-buffered at pH 6.8, is most probably a consequence of acetate toxicity. Previously we have noticed that a similar drop in the γ -actin (*acnA*) transcript level occurs between 36 and 40 mM in medium set at pH 6.8 (but not phosphate buffered) and that this effect occurs at increasingly lower acetate concentrations with decreasing medium pH (data not shown).

Transcription pattern of *acpA* upon addition of various weak carboxylic acids

To further specify the induction spectrum of *acpA* we performed northern blot analysis on RNA isolated from wild-type mycelia supplied with 7.2 mM (final concentration) of various weak monocarboxylic acids: lactate, pyruvate, propionate, formate and glyoxylate. Of these, only addition of propionate results in significant induction of *acpA* (Fig. 5). Interestingly, this induction is much weaker when propionate is supplied at 36 mM (data not shown), suggesting that the inducing ability of this weak acid is similar to that of acetate (*i.e.* it provokes transcriptional induction of *acpA* when present in limiting amounts). While growth was extremely poor on these monocarboxylic acids as sole carbon sources, no significant differences in growth were observed between the Δ *acpA* null mutants and the wild-type (data not shown). Moreover, testing propionate toxicity using a wide range of propionate concentrations on glycerol minimal medium plates (at pH 6.8) did not discriminate *acpA*-deleted strains from wild-types (data not shown). These data suggest that AcpA has a restricted substrate range and that acetate is its physiologically relevant substrate.

Transcriptional activation of *acpA*

Intriguingly, while the *acpA* response to glyoxylate was rather weak at low concentrations (Fig. 5) this intermediate of acetate catabolism provoked a powerful induction at 50 mM (data not shown) — the reverse of that observed for acetate and propionate. It has previously been shown that glyoxylate at 50 mM is also good inducer of the transcription of the acetyl-CoA synthetase gene *facA*, one of the structural genes under the control of the acetate pathway-specific activator FacB [27]. These observations prompted us to investigate whether the induction of *acpA* is dependent on the function of FacB. To address this we compared the expression of *acpA* between a wild-type strain and a *facB* mutant (*facB101*) after induction with ethanol and ethylacetate and used *acuE* as an expression control. The lack of *acpA* induction (Fig. 6) in the complete loss-of-function mutant *facB101* [28] correlates with the presence of six putative binding sites for the binuclear zinc-cluster protein FacB [29] in the promoter of the *acpA* gene: two sites located upstream of the distal transcriptional start and four more located upstream of the proximal start site (Fig. 1A). The induction pattern of *acpA* in a *facA* mutant (*facA330*) — the latter cannot convert acetate into acetyl-CoA — is similar to that observed in the wild-type strain, indicating that the loss of induction in the *facB101* mutant is due to the absence of functional FacB and not a consequence of the loss of the ability to catabolize acetate. It should be noted that our analysis suggests that the basal level (*i.e.* non-induced) expression of both *acpA* transcripts does not require FacB.

acpA is essential for acetate permease activity

To assess the role of *acpA* in acetate transport the uptake of ^{14}C -labelled acetate was compared between wild-type and $\Delta acpA$ null mutant conidia germinating in the presence of either glucose or ethanol as sole carbon sources, culture conditions known to result in *acpA* expression (data not shown). As can be seen in Fig. 7 acetate uptake by $\Delta acpA$ conidia is very considerably reduced compared to that of wild-type conidia. This is especially true at the lower acetate concentrations where the differences in calculated uptake rates range from 30 fold at 1 mM acetate to >100 fold at acetate concentrations between 1 – 100 μM . To characterise the kinetics of acetate uptake the incorporation of ^{14}C -acetate by wild-type conidia germinating in glucose at pH 6.8 was measured across a wide range of substrate concentrations (1 μM – 7 mM). Rates of uptake were obtained by linear regression of plots of acetate incorporation versus time. Rates plotted against substrate concentration showed Michaelis-Menten saturation kinetics from which a K_m of ~ 230 μM was calculated (Fig. 7 inset). Eadie-Hofstee and Hanes-Wolf representations of the data were monophasic hence indicating that no additional system of acetate incorporation contributed significantly to its

uptake. These data are consistent with the growth phenotype of the $\Delta acpA$ mutants on limiting amounts of acetate being due to an impairment of the uptake of the acetate anion, and the likelihood that AcpA functions as an acetate permease in *A. nidulans*.

DISCUSSION

In this paper we identify *acpA* (Broad annotation: AN5226) to encode a novel element in the biochemical pathway for acetate utilization in the filamentous fungus *Aspergillus nidulans*. The *acpA* gene is located on chromosome V but it is not closely physically linked to any of the five other loci involved in acetate metabolism that also reside on this chromosome: *facA* (AN5626), *acuD* (AN5634), *acuG* (AN5604), *acuH* (AN5336) and *acuN* (AN5746) [3]. Analysis of the sequence of the deduced translation product (AcpA) is indicative of a membrane related function since it bears homology to the defining members of the GPR1/FUN34/YaaH family (TC-9.B.33; Pfam PF01184) of membrane proteins [12, 30, 31], and its hydrophobicity profile indicates the presence of six transmembrane domains.

Unlike other activities involved in acetate utilization, AcpA is dispensable for growth on the acetate precursor ethanol. Whilst this is also largely the case when 1% acetate is used as the sole carbon source, at reduced concentrations the growth of AcpA loss-of-function mutants ($\Delta acpA$) is seen to become progressively impaired and this effect is further exacerbated at elevated pH. Previous studies of acetate uptake kinetics in yeast have provided evidence for distinct means of uptake for each of the two forms of acetate (*i.e.* HA and A⁻). Entry of the anion into the cell is mediated by a transport system whilst the undissociated acid is able to freely diffuse across the plasma membrane [32, 33]. The very poor growth observed for *A. nidulans* $\Delta acpA$ mutants on acetate under conditions in which the availability of the protonated (HA) form is very reduced or negligible (*i.e.* low acetate concentration and pH values considerably higher than the pKa) could thus be indicative of a defect in the transport of the anionic form. This is coherent with the observation of normal growth of $\Delta acpA$ mutants on ethanol since acetate is generated intracellularly upon catabolism of ethanol.

In addition to the growth phenotype on acetate, deletion of *acpA* results in altered regulation of the malate synthase (*acuE*) gene. Whilst *acuE* inducibility upon growth of $\Delta acpA$ mutants on acetate precursor substrates is unchanged from that of wild-type, its induction by limiting amounts of externally supplied acetate (0.1% at medium pH 6.8) is absent. Furthermore, the *acpA* transcript level in wild-type strains increases as the external acetate concentration decreases, *i.e.* the gene's response coincides with the need for a means to take up the acetate anion. These results are all consistent with the loss of an acetate uptake

function in the *acpA* deletion mutant. Indeed, direct measurement of acetate incorporation into germinating conidia confirmed the effect of the *acpA* deletion on acetate uptake. At low substrate (acetate) concentrations acetate uptake is almost completely lost in *acpA*-deleted conidia compared to that seen in wild-type.

It has previously been postulated [34] that a gene (GenBank accession AY236409) corresponding to the *A. nidulans* Broad locus *AN2840* could encode a monocarboxylate transporter (MCT) based on its similarity to functional mammalian MCTs. However, in *S. cerevisiae* of the five proteins that show substantial similarity to human MCT transporters none is important for the transport of carboxylic acids across the plasma membrane, and most are instead found associated with intracellular structures [35]. To our knowledge, AcpA is thus the first experimentally supported candidate for a MCT in filamentous fungi.

By reciprocal TBLASTN screening we searched for AcpA orthologues in publicly available fungal genome databases. In all cases except that of the *Chytridiomycota* at least one putative orthologue of *acpA* could be identified. Fig. 8 shows a phylogenetic tree of those proteins most similar to AcpA in 27 fungi. The proteins identified in the *Aspergilli* share 61% identity and 74% similarity. These data indicate conservation of AcpA-like proteins and by inference the means of direct cellular uptake of the acetate ion.

With regard to their structural organisation, all *Aspergillus spp acpA* orthologues appear to contain canonical splicing sites [36] in their 5' UTR sequences that encompass a region highly enriched in A residues (for *A. nidulans* see Fig. 1A nucleotide position -72 to -35). The apparent occurrence of an intron upstream of the coding region in all *Aspergilli acpA* orthologues suggests not only structural conservation within the 5' UTR sequences but also a specific function for the intron. A similar organization is known for the *A. nidulans* gene *brlA* which encodes a key regulator of asexual development. The molecular basis for the production of two distinct *brlA* transcripts and translation products has been studied [37, 38]. In the case of *acpA* however, the absence of any ATG in the 5' UTR of the longer transcript would suggest that both mRNA species probably give rise to the same translation product. This raises the question of the roles of the two *acpA* transcripts. One possibility can be illustrated by the *S. cerevisiae JEN1* gene [39]. For this gene, one of the several different transcription products that result from multiple initiation transcription sites was shown to modulate the degradation of all *JEN1*-specific mRNAs [40]. Another gene with an intron in its 5'UTR is the arginase encoding gene *agaA* of *A. nidulans* [41]. This intron leads to the generation of various mRNA isoforms that influence not only mRNA stability but also their

activities as templates for translation by a riboswitch mechanism [42]. However, unlike *acpA*, the different *agaA* mRNA species result from alternative splicing.

In *S. cerevisiae* two functional MCTs have been described: a high-affinity transporter shared by lactate, acetate, propionate and pyruvate [43], and a second transporter of lower affinity and more restricted substrate specificity since it only transports acetate, propionate and formate [32]. While the former was found to be Jen1p (TC-2.A.1.12.2) [39, 44] a membrane protein that is assigned to the Major Facilitator Superfamily (TC-2.A.1), the latter depends on the integrity of the yeast *acpA* orthologue, *ADY2* [11].

By screening the *A. nidulans* genome sequence [14] we identified two putative gene products similar to *S. cerevisiae* Jen1p encoded by loci *AN6703* and *AN6095*. The deduced proteins share 39% and 30% identity with Jen1p, respectively. Northern blot analysis upon addition of various monocarboxylic acids or ethanol to lactose-grown wild-type mycelia, failed to reveal expression of *AN6095* (unpublished data). By contrast, *AN6703* appeared to be modestly expressed upon formate and propionate induction but no response could be observed upon addition of acetate to 7.2 mM or 36 mM (0.1% - 0.5%) nor in the presence of ethanol (*i.e.* under conditions inducing for the *acpA* gene) (unpublished data). These observations would explain why conidia of the null $\Delta acpA$ mutant germinated on glucose or ethanol are barely able to take up acetate under our experimental conditions (medium pH of 6.8 or higher — see Fig. 7). Alternative explanations could be that *AN6703*, the *A. nidulans* protein most similar to the yeast transporter Jen1p, is either unable to transport acetate or — as is the case for ethanol — it is not expressed on glucose.

Together with previous yeast studies [11-13], our data demonstrate a link between members of the GPR1/FUN34/YaaH protein family (TC-9.B.33) and acetate utilisation, suggesting that other members of this family, including *A. nidulans* AlcS and orthologues in other filamentous fungi, might be involved in acetate adaptation, uptake and/or catabolism.

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Table 1. *A. nidulans* strains used in this study.

| Strain | Genotype | Source or reference |
|--------|---|------------------------|
| BF107 | <i>yA2 pabaA1, aldA67</i> ¹ | [45] |
| CV061 | <i>wA3, pyroA4, facA330</i> ^{4,5} | [3] |
| CV160 | <i>yA2 pabaA1</i> ¹ | Dr. Henk van den Broek |
| CV164 | <i>pabaA1, panB100, riboB2</i> ^{1,2,3} | This work |
| CV171 | <i>pyrG89 pabaA1, panB100, riboB2; Tr. ΔalcS::A.f. pyrG</i> ^{1,2,3} | This work |
| CV253 | <i>pyrG89 pabaA1, panB100, riboB2; Tr. ΔalcS::A.f. pyrG, ΔacpA::panB</i> ^{1,2,5} | This work |
| CV260 | <i>pabaA1, panB100, riboB2; Tr. ΔacpA::panB</i> ^{1,2,5} | This work |
| CV312 | <i>pabaA1, panB100, riboB2; Tr. panB</i> ^{1,2} | This work |
| CV313 | <i>pyrG89 pabaA1, panB100, riboB2; Tr. ΔalcS::A.f. pyrG, panB</i> ^{1,2} | This work |
| ED027 | <i>wA3, riboB2 facB101</i> ^{2,5} | [46] |

¹ *Para*-Amino Benzoic Acid auxotroph; ² Riboflavin auxotroph; ³ D-Pantothenic acid auxotroph; ⁴ Pyridoxine auxotroph; ⁵ Growth defect on acetate (0.1%).

A.f.: *Aspergillus fumigatus*; Tr: 'transformed with'.

Table 2. Oligonucleotides used in this study.

The shift from lower case to upper case corresponds to the junction between sequences from *acpA* and *panB*.

| Name | Sequence (5'->3') |
|-----------------------|---|
| 5226-5'anti | CTCCCGTGTGGTCAGCGACTAATTTCCC |
| 5226-5'- pto3'-sht | cgcgtgcctattctcatccaacacttcttcGGAGATCGAAGAGGAAGAGGCTTAGCTCG |
| 5226-3'sens | CGACCTGACGGATCTCGTCTGGTACGC |
| 5226-3'- pto5'-sht | ggcaatgcaagagatgtgaagaagtaagcgCGTGGAGCACAGTCAACCACTTGCAGAT |
| pto3'-5226- 5'sht | cgagctaagcctcttctcttcgatctccGAAGAAGTGTTGGATGAGAATAGGCACGCG |
| pto5'-5226- 3'sht | atctgcaagtgggtgactgtgetccacgCGCTTACTTCTTCACATCTCTTGCATTGCC |
| Nes5226- 3'sens | CGAGTGCTACTATAACACGG |
| Nes5226- 5'anti | CCGTCTCAGTTTCAAGCACG |
| RACE 5226- 5'anti | GTGTTTCCAACGGCCATTTCCCAC |
| RACE 5226- 3'sens | CTCGTTCGGTTTGTTCCTCATGGGC |

Figure legends

Figure 1: Structure of the *A. nidulans acpA* gene and its transcription and translation products.

(A) The *acpA* gene corresponds to the predicted ORF AN5226.3 on chromosome V in the *A. nidulans* genome. The sequence of *acpA* is available under GenBank accession number EF428327. All distances are relative to the first base of the presumed start codon for the AcpA protein (+1). The putative FacB-binding sites (class A consensus: TCC/GN₈₋₁₀C/GGA; class B consensus: GCA/CN₈₋₁₀T/GGC) [29] are indicated by dotted box and solid boxes, respectively. The two transcription starts and the major polyadenylation site are indicated by a grey background and labelled '5'L', '5'S' and '3'', respectively. The intron within the 5' UTR is indicated by a grey background. Inside this intron, the ATG and Stop codons that bound an ORF are underlined. The two introns disrupting the coding region are marked A and B. The deduced 298 residue sequence of the AcpA protein is given below the coding sequences. The six 21 amino acid long transmembrane domains predicted by the TopPred algorithm [47] (parameter settings: full window size 21, core window size 13, wedge window size 4) are indicated by the grey background. (B) Schematic representation of the two transcripts of *acpA*. The thick line represents genomic DNA while thin lines represent the two mRNA species. DNA coordinates are relative to the first base of the presumed start codon of the AcpA protein (+1). The positions of the two transcription start sites are marked '5'L' and '5'S'. Probes A and B were used for northern experiments. (C) Northern experiments were realized with RNA isolated from a $\Delta alcS$ strain (CV171) induced with 50 mM ethylacetate (see Experimental). The blots were subsequently hybridised with the appropriate ³²P-labelled probes (see Fig. 1B).

Figure 2: The *acpA* gene is required for growth on acetate minimal medium plates.

Plates with appropriately supplemented minimal medium were prepared containing sodium nitrate (10 mM) as nitrogen source and sodium acetate at the indicated concentration as the carbon source. The media were buffered at the indicated pH with 100 mM sodium phosphate (final concentration). Inoculated plates were incubated at 37°C for 2 days. The relative availabilities of the protonated (HA) and anionic (A⁻) forms are given by the [HA]:[A⁻] ratio for each pH. (A) Plates were point inoculated with four strains which differ only in the indicated deletion: the single mutants $\Delta acpA$ (strain CV260) and $\Delta alcS$ (strain CV313), and the double mutant $\Delta acpA\Delta alcS$ (strain CV253); strain CV312 was used as the control (see Table 1).

Figure 3: Malate synthase gene (*acuE*) transcription in the presence of a limiting amount of acetate depends on the integrity of the *acpA* gene. Fungal biomass was generated in lactose/urea liquid media and gene expression was induced/repressed with the indicated compounds by addition to mycelial cultures to a final concentration of 7.2 mM. Media were buffered at pH 6.8 with 100 mM sodium phosphate. Total RNA was isolated, denatured, separated and transferred to a nylon membrane as described in Experimental. Northern blots were subsequently hybridised with ³²P-labelled probes corresponding to the indicated genes (see Experimental). The γ -actin gene (*acnA*) served as an internal control. CV260 provided the Δ *acpA* mutant background and CV312 served as the wild-type strain (see Table 1). (A) For induction, acetate was added to the mycelial cultures to a final concentration of 7.2 mM (0.1%). The control, NI, received no inducer compound. (B) The same experiment was carried out except that ethanol or ethylacetate were added for induction instead of acetate.

Figure 4: Induction of *acpA* is inversely correlated to the acetate concentration. Experimental details are as described in the legend to Figure 3. Gene expression was induced with acetate across a range of concentrations from 7.2 mM (0.1%) to 36 mM (0.5%). The wild-type strain used was CV312 (see Table 1).

Figure 5: *acpA* expression in the presence of various weak monocarboxylic acids. Experimental details are as described in the legend to Figure 3. The indicated compounds were added to mycelial lactose cultures to a final concentration of 7.2 mM. The wild-type strain used was CV312 (see Table 1).

Figure 6: Involvement of the transcriptional activator of acetate catabolism FacB in *acpA* induction. Experimental details are as described in the legend to Figure 3 except that the final concentration of ethanol or ethylacetate was 50 mM. Strains ED027 and CV061 provided *facB* and *facA* mutant backgrounds, respectively. CV160 was the wild-type strain used.

Figure 7: AcpA is required for acetate uptake at pH 6.8. Acetate uptake by WT (CV312) or Δ *acpA* (CV260) conidia germinating in liquid glucose minimal medium (see Experimental) was determined at different concentrations of acetate (1, 10, 100, 1000 and

10,000 μM). Inset: Michaelis-Menten plot of acetate uptake rate versus substrate concentration for the wild-type strain. Uptake rates were measured for eighteen different concentrations of acetate. V° is expressed in nmol of acetate taken up per 5×10^7 conidia per second. The solid line represents the best fit for the data. Representative data from one of five independent experiments are shown.

Figure 8: Neighbour-joining phylogenetic analysis of fungal AcpA orthologues. AcpA orthologue proteins were identified by TBLASTN screening of the genome sequence data of the following species: *Neosartorya fischeri*, *Aspergillus fumigatus*, *Aspergillus clavatus*, *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger*, *Uncinocarpus reesei*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Ascospaera apis*, *Botryotinia fuckeliana*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Septoria nodorum*, *Chaetomium globosum*, *Podospora anserina*, *Neurospora crassa*, *Fusarium verticillioides*, *Fusarium graminearum*, *Trichoderma reesei*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Ustilago maydis*, *Coprinus cinereus* and *Rhizopus oryzae*. A sequence alignment was generated using the CLUSTALW multiple sequences alignment program [48] and the PHYLIP package [49]. The tree was rooted using protein sequence RO3G_03025 of *Rhizopus oryzae* as an outgroup. The bootstrap probabilities (calculated using 100 replications) are indicated on each node. For those organisms where a database of deduced proteins was accessible, the corresponding protein annotation code is given (provided that the predicted protein is identical to that we deduced by manual analysis).

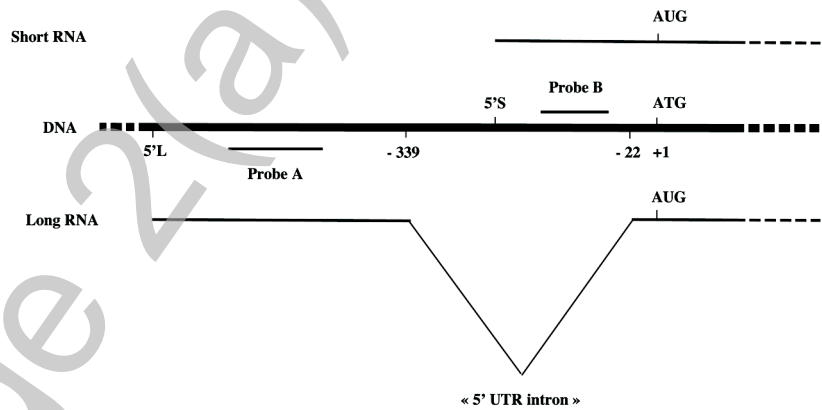
FIGURE 1

A

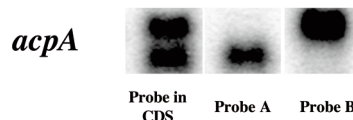
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E F Q P G L Y K S V E A R K F A N P A P L G L S A F A L T T F V L S C I N M G A AA107
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R D I T H P N I V I A L A F G Y G G L V Q L L A G M W -----Intron A----- AA134
tcagccaggaagctaatcatgagcagGAAATGGCCGTTGGAAACACTTTTGGTGCACCTGCTCTCTCTTATGGTGGTTTCGGATTGCGTTCGCCATTTGCTTACTCCCGGTGT -560
E M A V G N T F G A T A L S S Y G G F W I A F A I V L T P G G AA165
TTCAACATTAGACCCGCTCACGGCTGAAATGGTATGAGGCCATGTTCTACAACCTGCTCGGTTTGTCTCATGgtgagtggttccatcaatcagcatgaccggttcggcgtgac -680
F N I Q T A L T A E N G D E A M F Y N S F G L F L M -----Intron B----- AA191
aatgtcccagcggTGGTTCATCTTACCACCATCATGCTTTTCTGCACCTGAGGTCTACCGTCGCCTTCTCTGCTGTTCTGTTCTCTCGACCTCGCGTTCCTGCTCTCCGGTGTG -800
G W F I F T T I M L F C T L R S T V A F F L L F L F L D L A F L L L G V G AA227
GTTACATCCAGCGTGATGATGCTGGCCAGCCCAACCTCCTGTCATCAAGCCGTTGGTTTCTTGGTCTGCTGCCTTCTGCTTGGTACAACGCCCTTCCCGGTATTGCCGACA -920
Y I Q R D D A G Q P N P P V I K A G G F F G L L A A F A A W Y N A L A G I A D S AA267
GCAGCAACAGCTTCTCATCATCCCTGTGCCCACTTCCCGTGGTCCCTACCGGTCTGCTGCTGAGAGACCGGAGCGGAGACTGTTTAacccctcgtctcgcgctctcgtca -1040
S N S F F I I P V A H F P W S P T G R A R R E K T E R E T V * AA298
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B

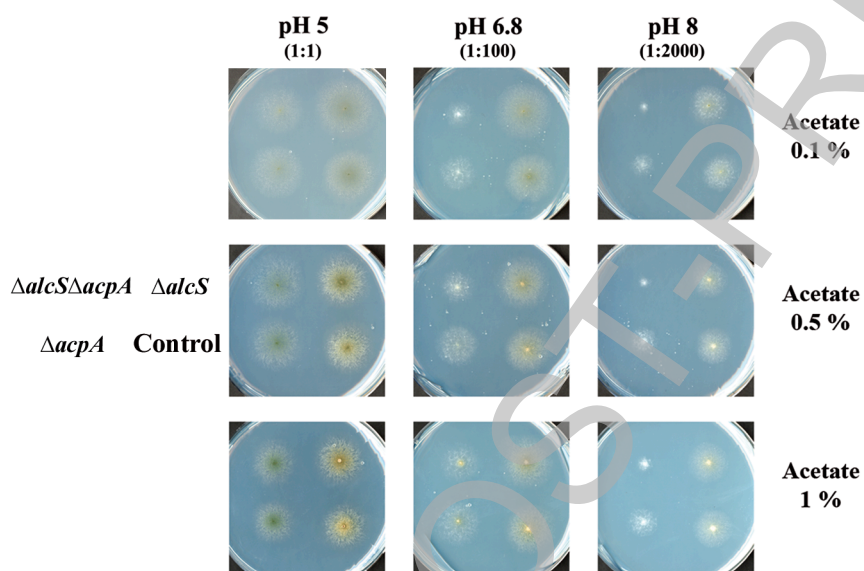


C



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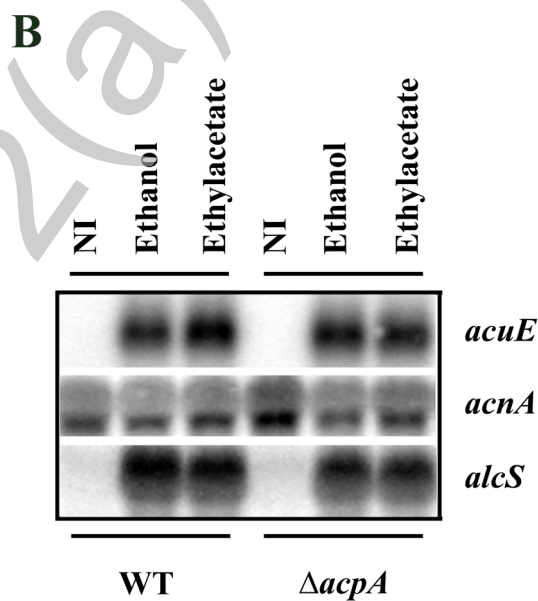
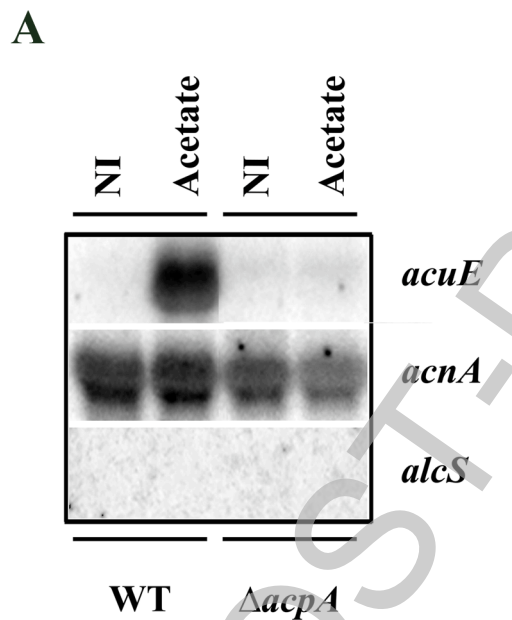
FIGURE 2



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Stage 2(a) POC PRINT

FIGURE 3



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FIGURE 4

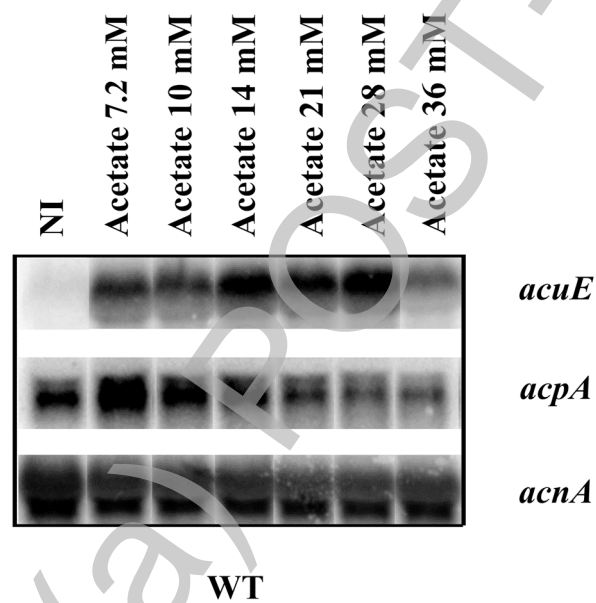
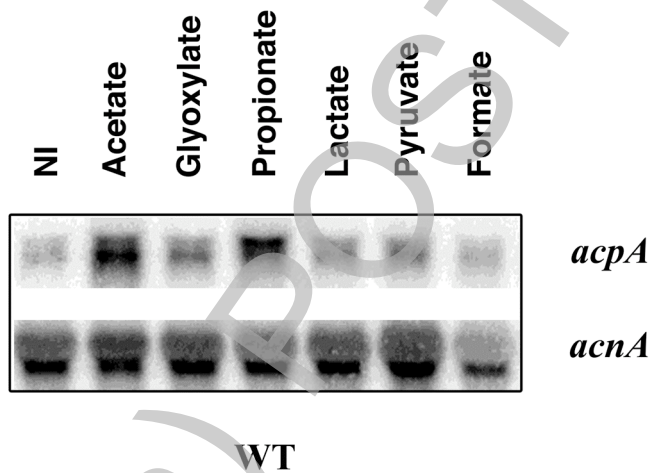


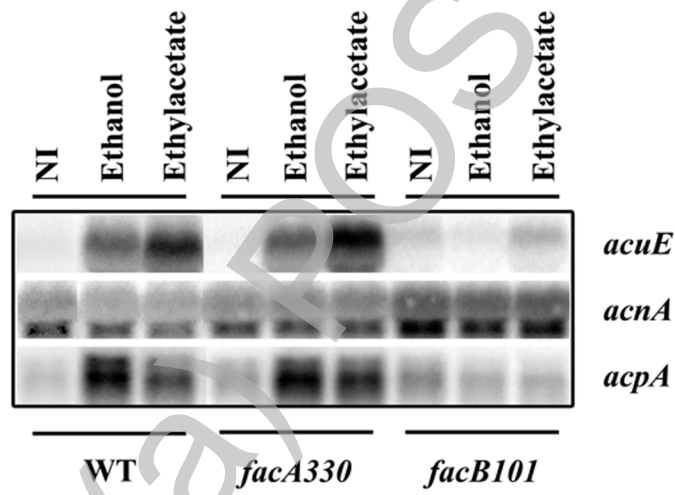
FIGURE 5



Stage 2(a) WT

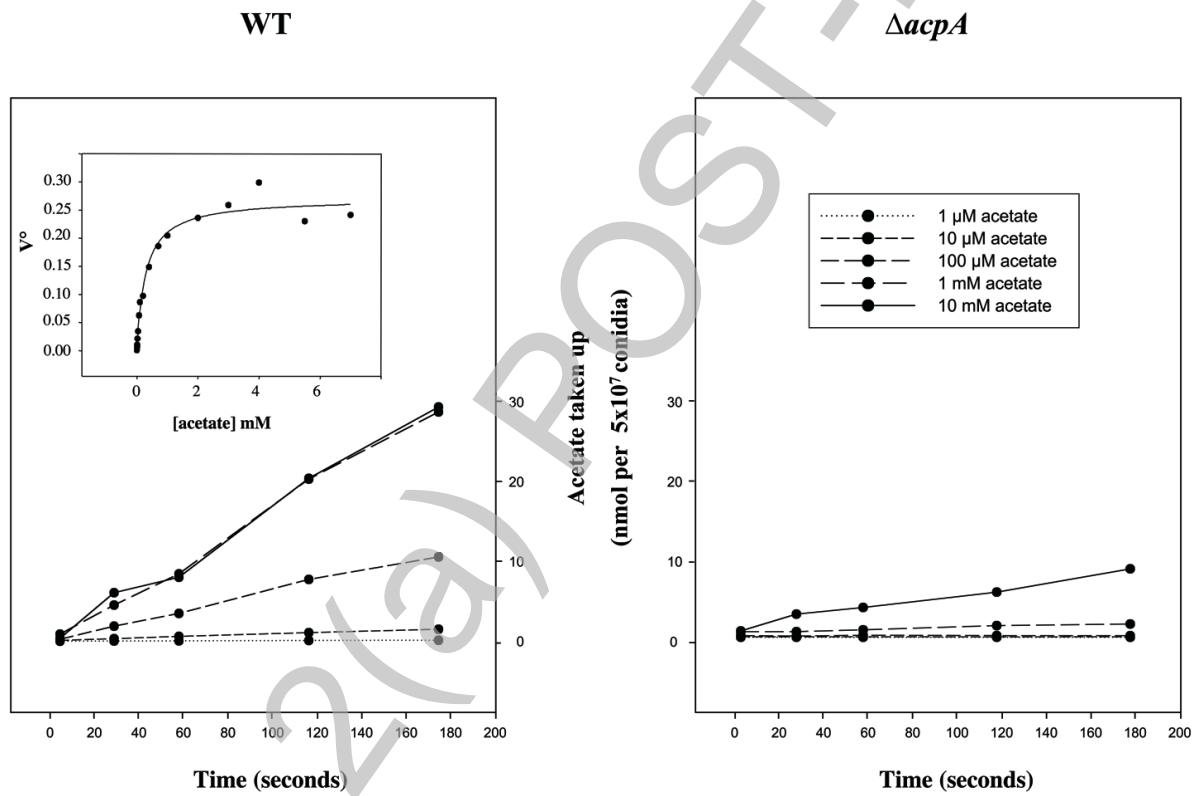
THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080124

FIGURE 6



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FIGURE 7



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FIGURE 8

